Centro de Investigación en Alimentación y Desarrollo, A.C.

EVALUACIÓN DE LOS FACTORES RESPONSABLES DE LA ACTIVIDAD ANTIOXIDANTE Y ANTIMICROBIANA DE LOS PROPÓLEOS CON POTENCIAL UTILIZACIÓN EN LA CONSERVACIÓN DE PRODUCTOS CÁRNICOS

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y hermanos (Mayra Armida, Yeimi Rocío y Raúl Crisanto),

quienes han sido mi más grande

apoyo e inspiración"

CONTENIDO

RESUMEN

El propóleos es una sustancia compleja producida por las abejas (*Apis mellifera*) que contiene principalmente resinas, ceras y polen, materiales colectados de diferentes secreciones de las plantas. En las últimas décadas, el propóleos ha ganado una amplia aceptación en diferentes países. A partir del propóleos se obtienen extractos etanólicos (EEP), los cuales se caracterizan por poseer diversas propiedades biológicas, destacando su actividad antioxidante (AOX) y antimicrobiana (AMI), las cuales son dependientes de la época de colecta, origen geográfico y botánico, así como de la presencia de polifenoles. Sin embargo, existen diversos aspectos de este subproducto apícola que no se han explorado, tales como: (i) el comportamiento del EEP en productos cárnicos, (ii) la fuente botánica de los propóleos del Desierto de Sonora, (iii) la relación existente entre el origen botánico y la AOX y AMI del EEP, (iv) la relación existente entre los parámetros fisicoquímicos, organolépticos y propiedades biológicas con las coordenadas de color de muestras de propóleos en crudo y, (v) a través de métodos computacionales (Teoría de Funcionales de la Densidad) estudiar las propiedades estructurales, de potencial y reactividad química, así como los mecanismos de inhibición de radicales libres (HAT, SPLET y SET-PT) involucrados en la AOX de los compuestos polifenólicos. Por lo anterior, el objetivo general de este trabajo de investigación fue caracterizar física, química, fisicoquímica, organoléptica, biológica y botánicamente los propóleos, para establecer su potencial aplicación en productos cárnicos.

Los resultados demostraron el potencial del EEP como ingrediente conservador en productos cárnicos durante su almacenamiento en refrigeración; además de encontrarse que las principales fuentes botánicas de los propóleos del Desierto de Sonora son "*Mimosa distachya,* var. *laxiflora*" conocida como Uña de gato y "*Prosopis velutina*" conocida como Mezquite. El origen botánico, las características fisicoquímicas y organolépticas del propóleos están altamente correlacionados con sus propiedades biológicas. Los compuestos identificados

del EEP fueron ácido gálico, ácido cinámico, ácido p-cumárico, naringenina, quercetina, luteolina, kaempferol, apigenina, pinocembrina, pinobanksina 3-acetato, éster fenetílico del ácido cafeico (CAPE), crisina, galangina, acacetina y pinostrobina. Los métodos computacionales permitieron: reproducir las estructuras de los compuestos de interés; establecer su potencial químico y propiedades de reactividad; evidenciar que el mecanismo de inhibición de radicales libres de los compuestos fenólicos, fue el HAT; y que CAPE fue el compuesto con mayor AOX. Los resultados muestran que además de los grupos-OH, los grupos-CH contribuyen en la AOX de polifenoles encontrados en el EEP.

Palabras Clave: Propóleos, antioxidante, antimicrobiano, origen botánico, mecanismos antioxidantes, productos cárnicos.

ABSTRACT

Propolis is a complex substance produced by honeybees (Apis mellifera) containing mainly, resins, waxes and pollen, materials collected from different secretions of plants. In recent decades, propolis has gained wide acceptance in different countries. Ethanol extracts (PEE) are obtained from propolis, which are characterized by different biological properties, highlighting its antioxidant (AOX) and antimicrobial (AMI) activity, which are dependent on the season of collection, geographic and botanical origin and the presence of polyphenols. However, there are several aspects of this beekeeping product, that has not been explored, such as: (i) behavior of the PEE in meat products; (ii) botanical origin of the Sonoran Desert propolis; (iii) relationship between the botanical origin with the AOX and AMI of EEP; (iv) relationship between the physicochemical and organoleptic parameters with the color coordinates of raw propolis samples; and (v) through computational methods (Density Functional Theory) study the structural properties, chemical potential and reactivity, and the mechanisms of free radical inhibition (HAT, SPLET and SET-PT) involved in the AOX of polyphenols compounds. Therefore, the main objective of this research was characterized physical, chemical, physicochemical, organoleptic, biological, and botanically the propolis, to stablish its potential application in meat products.

The results demonstrate the potential of PEE as preservative ingredient in meat products during chill storage; the main botanical origin of Sonoran Desert propolis was "*Mimosa distachya* var. *laxiflora*" known as Catclaw and "*Prosopis velutina*" known as Mesquite. The botanical origin, physicochemical and organoleptic characteristics of propolis are highly correlated with their biological properties. The phenolic compounds identified in PEE were Gallic acid, cinnamic acid, *p*-coumaric acid, naringenin, quercetin, luteolin, kampferol, apigenin, pinocembrin, pinobanksin 3-acetate, caffeic acid phenethyl ester (CAPE), chrysin, galangin, acacetin and pinostrobin. Computational methods allowed reproduce the structures of compounds of interest; establish its chemical

potential and reactivity properties; evidence that the free radical inhibition mechanisms, was the HAT; and CAPE was more AOX compound. Results also showed that besides OH-groups, the groups-CH contribute to the AOX of polyphenols found in PEE.

Keywords: Propolis, antioxidant, antibacterial, botanic origin, antioxidant mechanism, meat products.

SINOPSIS

Desde épocas muy remotas los productos de origen natural y sus derivados han sido empleados como agentes terapéuticos en la medicina tradicional. Actualmente, diversos estudios científicos los catalogan como una fuente prometedora para el desarrollo de nuevos conservadores alimentarios. Las reacciones de oxidación de lípidos y proteínas, así como la contaminación bacteriana, son tres de las principales causas que determinan la pérdida de la calidad y aceptación de los alimentos, como es el caso de la carne y productos cárnicos, durante su almacenamiento y procesamiento. En conjunto, estos factores generan cambios en el color, olor, sabor, valor nutritivo y textura, además de provocar daños en la salud del consumidor al momento de ingerir estos productos en estado de descomposición.

El incremento de la demanda de productos cárnicos frescos, genera que la industria desarrolle diversas tecnologías de conservación de la carne fresca: altas presiones hidrostáticas (HHP), sistemas de empaque de atmósfera modificada (MAP), empaques activos (AP), así como la incorporación de aditivos antioxidantes y antimicrobianos de origen sintético o natural. Una de las formas más empleadas para preservar alimentos ha sido el uso de aditivos sintéticos como butil hidroxianisol (BHA), butil hidroxitolueno (BHT) y terbutil hidroxiquinona (TBHQ). Sin embargo, diversos estudios ponen de manifiesto que estos compuestos son nocivos para la salud, por lo que, una de las formas más novedosas para la conservación de alimentos es el uso de extractos de origen natural con denominación GRAS (generally recognized as safe, por sus siglas en inglés), con propiedades biológicas tan eficaces como los aditivos comercialmente disponibles.

Algunos extractos de origen natural como el cacao, arroz, frijol, ciruela, manzana, uva, cereza, cebolla roja, orégano, romero, comino, chile, soya, miel, jalea real y propóleos, han sido reconocidos por poseer ciertas propiedades biológicas, capaces de inhibir las reacciones de oxidación de lípidos y proteínas, asi como reducir la existencia de ciertos microorganismos patógenos, debido a la presencia de compuestos fenólicos, a saber: ácidos fenólicos y flavonoides. El propóleos es una sustancia de composición compleja elaborada por las abejas (*Apis mellifera*), a partir de resinas de ciertas plantas que las abejas modifican por glucólisis con enzimas de las glándulas de la hipofaringe, las cuales posteriormente son mezcladas con cera y polen. Esta sustancia es usada como un protector natural contra microorganismos patógenos y hongos; protege contra el frío durante el invierno y actúa como material de sellado en las paredes externas e internas de la colmena para reducir la entrada de insectos. Sus principales componentes son resinas (50-55%), ceras (25-35%), aceites volátiles (10%), polen (5%), minerales y sustancias orgánicas (5%).

Este producto es muy apreciado por su actividad biológica: antibacteriana, antiviral, antifúngica, anticancerígena, antioxidante, cicatrizante, inmunoestimulante, anestésica, analgésica y fitoinhibidora, entre otras. Tomando en consideración esta información, se establece la siguiente hipotesis de investigación: las propiedades físicas, químicas, fisicoquímicas, organolépticas y biológicas, así como el origen botánico de los propóleos, determinan su potencial uso como conservador en productos cárnicos.

En la primera etapa (**Capítulo I**) de este trabajo de investigación se estableció como objetivo hacer una revisión de literatura, acerca de las posibles aplicaciones de extractos de propóleos realizadas en diversas matrices alimentarias, para establecer el potencial de utilización de los extractos de propóleos como aditivos en productos cárnicos. En base al material encontrado en este capítulo, se concluye que los extractos de propóleos sí pueden ser

incorporados en matrices alimentarias, debido a las diversas propiedades biológicas que ha presentado en otros alimentos de origen animal y vegetal.

En el **Capítulo II**, se evaluó el efecto de los extractos etanólicos de propóleos (PE) y antioxidantes sintéticos (butilhidroxitolueno, BHT; ácido ascórbico, Asc ac) como inhibidores de la oxidación de lípidos y proteínas, y estabilizadores de los cambios del color en hamburguesas de bovino y de cerdo sin cocinar, almacenadas en condiciones de refrigeración (2 °C/9 días de almacenamiento/en oscuridad). Los resultados de este estudio indicaron que la incorporación de los extractos etanólicos de propóleos en las hamburguesas, redujo significativamente ($P<0.05$) la oxidación de lípidos y proteínas, así como la pérdida del color rojo característico de la carne fresca. Los resultados demostraron el gran potencial de los extractos de propóleos como ingrediente en la industria de la carne para extender la vida de anaquel durante el almacenamiento en refrigeración.

Actualmente, las normas internacionales (Norma IRAM-INTA 15935-1) autorizan el uso del propóleos como ingrediente únicamente en productos como caramelos, mieles, y extractos a base de propóleos, ya sea en solución hidroalcohólica, de etanol o propilenglicol, además de ciertos suplementos dietéticos (**Capítulo I**). Sin embargo, a pesar de haber demostrado la actividad antioxidante presentada por los extractos de propóleos en productos cárnicos (**Capítulo II**), es importante tomar en cuenta que diversos estudios y normas internacionales consideran que para que el propóleos pueda ser utilizado como aditivo alimentario, debe cumplir con ciertos requisitos de calidad, incluyendo aspectos organolépticos, fisicoquímicos, composición química, propiedades biológicas y fuente botánica. Todas estas características son altamente variables y dependen de la ubicación geográfica, época del año o condiciones climáticas.

Por lo anterior, en este trabajo se abordó como etapa siguiente establecer el origen botánico de las muestras de propóleos a través de un análisis

melisopalinológico, para establecer el perfil polínico de las muestras (**Capítulo III**). Por ello se colectaron muestras de propóleos en las comunidades de Pueblo de Álamos y Rancho Viejo (Ures, Sonora; 29.1476 N, -110.1239 O; 632 m) en dos épocas del año (invierno y verano) durante 2012 y 2013. Además, se realizó un análisis de la vegetación dominante en la zona para conocer las plantas posiblemente visitadas por las abejas para la obtención de los materiales con las cuales elaboran la miel, cera y propóleos. Los resultados indicaron que en las muestras analizadas se encontraron un total de 42 tipos de polen, considerandose como fuente polínica principal (15-45%) en la región de Pueblo de Álamos a "*Mimosa distachya* var. *laxiflora*", planta conocida como "uña de gato"; mientras que en la región de Rancho Viejo a "*Prosopis velutina*", comunmente denominada "Mezquite", las cuales son plantas características y de alto valor, para animales y habitantes del Desierto Sonorense. Los resultados indicaron que estas plantas contribuyen de manera importante a la formación de los propóleos evaluados.

Algunos investigadores, correlacionan los aspectos organolépticos, fisicoquímicos, composición química y actividad biológica de los extractos de propóleos Sonorenses con la época y fuente botánica de la cual la abeja colecta las resinas para formar el producto. Sin embargo, como se mencionó anteriormente tales resinas al ser digeridas enzimáticamente son mezcladas con material polínico, el cual es altamente rico en compuestos fenólicos y forma parte del 5% de la composición general del producto formado. Debido a lo anterior y aunado a la falta de conocimiento, en el **Capítulo IV,** se evaluó el efecto de la fuente polínica (*Mimosa distachya* var. *laxiflora* y *Prosopis velutina*) en las características organolépticas del propóleos en crudo (apariencia, color, aroma, sabor, consistencia e impurezas visibles) y fisicoquímicas (humedad, cenizas, ceras, resinas e impurezas mecánicas), así como la actividad antioxidante (contenido de fenoles totales, flavonas y flavonoles, flavanonas y dihidroflavonoles, poder reductor del hierro y actividad antiradical DPPH) y antimicrobiana (concentración mínima inhibitoria frente a patógenos

comúnmente encontrados en productos cárnicos) de sus extractos, elaborados a base de soluciones etanólicas. Los resultados del análisis multivariado (análisis de componentes principales, PCA) mostraron que los propóleos provenientes de *Prosopis velutina* fueron los que presentaron la mayor capacidad antioxidante (alto contenido de fenoles totales y flavonoides) y antimicrobiana frente a patógenos Gram-positivos (*S*. *aureus* y *L*. *innocua*); mientras que *Mimosa distachya* var. *laxiflora* presentó alta actividad frente a bacterias Gram-negativas (*E*. *coli* y *S*. *thyphimurium*), principalmente durante el verano, lo cual se asoció a la presencia de compuestos fenólicos tales como ácido gálico, ácido cinámico, ácido *p*-cumárico, naringenina, quercetina, luteolina, kaempferol, apigenin, pinocembrina, acetate de pinobanksina, CAPE, crisina, galangina, acacetina y pinostrobina. Respecto a las propiedades organolépticas y fisicoquímicas, el color y el contenido de resinas mostraron una alta correlación con la actividad antioxidante y antimicrobiana de los extractos. La información contenida en este capítulo deja en evidencia la importancia de considerar el material polínico como una de las propiedades a considerar en la evaluación de calidad de los propóleos.

En base a las normas internacionales (Norma Salvadoreña, 2003), se considera que el color subjetivo de los propóleos crudos es una de las características importantes al evaluar su calidad. Sin embargo, en base a una revisión literaria constante se definió que no existen estudios en los que se relacione el color instrumental de las muestras de propóleos crudas con sus propiedades fisicoquímicas y biológicas (actividad antioxidante y antimicrobiana). Por lo anterior, en el **Capítulo V** se consideraron como objetivos (i) determinar los aspectos fisicoquímicos de muestras de propóleos colectadas en diferentes zonas y estaciones (invierno y verano) en el Noroeste de México, (ii) determinar su actividad antioxidante y antimicrobiana, con la finalidad de correlacionarlas con las coordenadas de color, y (iii) establecer nuevos criterios de clasificación en base al color de este producto. Como parte de los resultados, se encontró

una importante correlación de las propiedades biológicas con los indicadores de color instrumental, principalmente con el parámetro h*.

Los capítulos anteriores (**Capítulos I-V**) resaltan y demuestran el potencial uso de los extractos de propóleos en matrices alimentarias, debido a su rica composición de compuestos fenólicos y la capacidad que estos tienen para ejercer propiedades antioxidantes y antimicrobianas. Sin embargo, debido a la compleja composición de compuestos de origen fenólico encontrados en extractos de propóleos, muchos de los mecanismos por los cuales se ejercen dichas propiedades, no han sido esclarecidos. En base a esto, en el **Capítulo VI**, se llevó a cabo una revisión de literatura sobre los posibles mecanismos involucrados en la actividad antioxidante y antimicrobiana de los extractos de propóleos. Sobre las bases de dicha revisión, para la actividad antioxidante se estableció que el mecanismo propuesto es el secuestro de radicales a través de la transferencia de átomos de hidrógeno de los grupos hidroxilo de los compuestos fenólicos presentes en los extractos, tales como ácidos fenólicos (ácido cafeico, ácido cinámico, ácido p-cumárico y ácido ferúlico, entre otros) y flavonoides (acacetina, apigenina, crisina, galangina, kaempferol, naringenina, pinobanksina, pinocembrina, quercetina, entre otros). Mientras que en relación a la actividad antimicrobiana, se encontró que los compuestos fenólicos están involucrados en dos mecanismos: inhibición de la síntesis de ácidos nucleicos y degradación de la membrana citoplasmática.

Debido a la gran diversidad de mecanismos por los cuales los compuestos de origen fenólico pueden actuar, es esencial el conocimiento del concepto de reactividad en una molécula, ya que permite comprender las interacciones que operan durante su mecanismo de reacción; sin embargo, obtener tal conocimiento de forma experimental, resulta complicado y tardado. Actualmente, una de las herramientas usadas para el cálculo de estas propiedades de reactividad es mediante Química Computacional, la cual se define como la "disciplina orientada al entendimiento de la química, que se crea a partir de la

combinación de principios matemáticos y leyes fundamentales de la física". Esta herramienta funciona de manera similar a lo que ocurre en el laboratorio; sin embargo, es traducido al lenguaje de la computadora. De los métodos computacionales que actualmente existen, el más utilizado, debido a su versatilidad (genera un balance entre tiempo-costo-precisión en los cálculos), es el denominado Teoría de Funcionales de la Densidad (DFT). Este método realiza cálculos de energía a partir de una ecuación análoga a la ecuación de onda de Schrödinger; no obstante, para realizar el cálculo de esta energía es necesario establecer una química modelo, y sus dos componentes principales (funcional y conjunto de base), con los cuales se crean los orbitales moleculares para obtener dicha energía de manera más precisa. Así, el objetivo del **Capítulo VII** fue estudiar las propiedades moleculares y de reactividad química de la pinocembrina, compuesto encontrado en altas concentraciones en extractos de propóleos y altamente asociada con sus propiedades biológicas; a través del estudio de su estructura molecular; así como determinar las propiedades de potencial químico y de energía, mediante el método DFT. Además, se comparó la precisión en los cálculos de diversas químicas modelo, con el propósito de contribuir a un mejor conocimiento de las propiedades intrínsecas de esta molécula. Los resultados de la estructura molecular de pinocembrina fueron comparados con datos experimentales, encontrándose que las diferentes químicas modelo, principalmente la M05-2X/6-31G(d,p), fueron capaces de reproducir la estructura molecular de este flavonoide. Los valores de potencial químico encontrados en la molécula de pinocembrina, fueron bajos, lo cual indica que este compuesto ejerce su acción antioxidante donando electrones. Mientras que en relación a las propiedades de reactividad, evaluadas a través del análisis de Fukui, se encontró que en todas las químicas modelo evaluadas el sitio preferido para el ataque nucleofílico fue el $C_{(4)}$; sin embargo, para el ataque electrofílico y radical, los grupos $O_{(2)}$ y $C_{(8)}$, fueron los sitios preferidos.

Finalmente en el **Capítulo VIII**, a través del método DFT, se estudió la relación de la estructura molecular con la actividad antioxidante de algunos de

los compuestos fenólicos mayoritarios, presentes en los extractos de propóleos crisina, galangina, pinocembrina, pinostrobina y CAPE). En el estudio se consideraron tres mecanismos de inhibición de radicales libres: (i) transferencia directa del átomo de hidrógeno (HAT, por sus siglas en inglés); (ii) transferencia secuencial del protón y el electrón (SET-PT); y, (iii) transferencia secuencial del electrón y el protón (SPLET). Los resultados mostraron que el mecanismo de acción dominante de las moléculas evaluadas fue el HAT; siendo la molécula de CAPE la que presentó los valores de energía de disociación de enlace más bajos, por lo que se puede considerar la de mayor actividad antioxidante. Otra aportación importante de los resultados es que los grupos carbono-hidrógeno (C-H), al igual que los grupos oxígeno-hidrógeno (O-H), juegan un papel importante en la actividad antioxidante de los compuestos fenólicos presentes en los extractos de propóleos.

HIPÓTESIS

Las propiedades físicas, químicas, fisicoquímicas, organolépticas y biológicas, así como el origen botánico de los propóleos, determinan su potencial uso como conservador en productos cárnicos.

Objetivo General

Caracterizar física, química, fisicoquímica, organoléptica, biológica y botánicamente los propóleos, para establecer su potencial aplicación en productos cárnicos.

Objetivos Específicos

1. Evaluar el efecto antioxidante de los extractos de propóleos en hamburguesas de bovino y cerdo sin cocinar, almacenadas en condiciones similares a las de comercialización.
2. Establecer el origen floral de los propóleos producidos en las comunidades de Rancho Viejo y Pueblo de Álamos (Ures, Sonora), durante la época de invierno y verano (2012 y 2013).
3. Determinar las propiedades físicas, químicas, fisicoquímicas y organolépticas de los propóleos colectados en las diferentes zonas.
4. Evaluar el efecto antioxidante (contenido de fenoles totales, actividad DPPH, poder reductor) y antimicrobiano (contra patógenos comúnmente encontrados en carne y productos cárnicos) *in vitro* de los extractos etanólicos de propóleos.
5. Caracterizar compuestos fenólicos (ácidos fenólicos y flavonoides) presentes en los extractos etanólicos de propóleos.
6. Analizar mediante metodologías computacionales las propiedades estructurales y de reactividad de compuestos fenólicos comúnmente encontrados en los propóleos.

A continuación se describe el cumplimiento de los objetivos mencionados previamente, organizando los resultados en forma de capítulos que contienen los artículos publicados y preparados para cada uno de los temas estudiados.

CAPÍTULO I

El Propóleos: conservador potencial para la industria alimentaria (2013).
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Artículo de revisión publicado

EL PROPÓLEOS: CONSERVADOR POTENCIAL PARA LA INDUSTRIA ALIMENTARIA

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RESUMEN

El propóleos es un producto de la colmena formado por resinas que las abejas recolectan de ciertas especies de plantas. Diversos estudios demuestran que posee propiedades antioxidantes, antimicrobianas y antifúngicas, entre otras, las cuales dependen de su origen botánico, composición química, estación climática, método de extracción, edad y zona geográfica de recolección. El propóleos por ser un producto natural recibe la denominación GRAS (Generalmente reconocido como seguro). En algunos estudios se ha demostrado el efecto de los extractos de propóleos sobre ciertas bacterias y hongos, así como patógenos de interés alimentario, además de la capacidad que tienen para prevenir o retardar reacciones de oxidación, lo cual los convierte en productos naturales potencialmente atractivos para ser utilizado como conservador alimentario en sustitución de los aditivos sintéticos. Se concluye que los extractos de propóleos pueden ser incorporados en matrices alimentarias debido a las diversas propiedades biológicas que presentan.

Los productos naturales son una fuente prometedora para el desarrollo de nuevos conservadores alimentarios. Actualmente, el estilo de vida ha ocasionado interés por parte de los consumidores, industriales e investigadores sobre lo que podría denominarse como el retorno a lo natural, buscando la forma de ayudar a mantener la salud humana. Ello trae como consecuencia la necesidad de buscar nuevas fuentes naturales de aditivos alimentarios con la denominación GRAS (del inglés *generally recognized as safe*), como una alternativa al uso de compuestos sintéticos (Fernández-López *et al*., 2005). En las últimas décadas han sido publicados varios estudios relacionados con la composición de productos apícolas (miel, polen, jalea real y propóleos) y sus propiedades biológicas, lo cual genera la atención de investigadores e industriales en el uso y desarrollo de este tipo de productos (Viuda-Martos *et al*., 2008).

El propóleos es un producto de la colmena formado por resinas que las abejas recolectan de ciertas especies particulares de plantas, en particular de flores y brotes de las hojas, las cuales mezclan con la saliva, enzimas y otras secreciones propias de las abejas (Farré *et al*., 2004). Es usado como un protector natural contra microorganismos patógenos y hongos; protege contra el frío durante el invierno y actúa como material de sellado en las paredes externas e internas de la colmena para reducir la entrada de insectos (Marcucci *et al*., 2001; Papotti *et al*., 2012). Este producto es muy apreciado por sus actividades biológicas: antibacteriana, antiviral, antifúngica, anticancerígena, antioxidante, cicatri-

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zante, inmunoestimulante, anestésica, analgésica y f itoinhibidora, entre otras. Estas características están relacionadas con su composición química, origen botánico, época de recolección y la especie de abeja recolectora (Manrique y Santana, 2008; Gregoris *et al*., 2011; Papotti *et al*., 2012). Al igual que la miel, el propóleos es conocido desde la antigüedad y ha sido utilizado por las culturas egipcia, griega, romana, maya e inca (Kumar *et al*., 2008). Durante los últimos 30 años se ha retomado su uso en diversos países como Argentina, Brasil, China, Japón y México (Mendes da Silva *et al*., 2006; Manrique y Santana, 2008; Chaillou y Nazareno, 2009; Yang *et al*., 2011; Valencia *et al*., 2012), para el tratamiento de diversos padecimientos, tales como diabetes, quemaduras, faringitis y úlceras estomacales, por lo que su principal destino ha sido la industria farmacéutica (Farré, 2004).

Para el apicultor, la recolección de propóleos no implica reducir el rendimiento en la obtención de otros productos como miel, polen, cera y jalea real, o la adición de algún coste específ ico; sólo es necesario considerar el tiempo empleado en su recolección y en su manejo antes de ponerlo a la venta, por lo que el apicultor podría obtener una fuente de ingresos adicional. En la actualidad hay más de 90 productos a base de concentrado de propóleos, tales como jabón de baño, champú, pasta dental, enjuague bucal, cremas faciales, cosméticos, pomadas, ampolletas y suplementos alimenticios. Sin embargo, su uso no ha sido explotado a nivel industrial y de investigación en alimentos (Kosalec *et al*., 2005; Gregoris *et al*., 2011). Debido a su composición diversa, el propóleos posee características muy complejas, por lo que su empleo a nivel industrial, trae como consecuencia la necesidad de establecer nor malización y controles de calidad, tomando como punto de par tida su origen botánico, composición química, propiedades biológicas, características f isicoquímicas y organolépticas, así como su toxicidad (Peña, 2008; Valencia *et al*., 2012), lo cual deter minará si es posible el uso de este valioso producto en alimentos. El objetivo de esta revisión es discutir la posibilidad del propóleos para ser utilizado como conser vador en la industria alimentaria.

Origen Botánico del Propóleos

El propóleos contiene una amplia variedad de compuestos químicos, habiéndose identif icado más de 300, entre los que se hallan polifenoles, ácidos fenólicos y sus ésteres, aldehídos, alcoholes, cetonas, ter penoides, esteroides, aminoácidos y compuestos inorgánicos (Nagai *et al*., 2003). Sin embargo, la composición química de este producto apícola es altamente variable y depende de la f lora local del sitio de recolección, tanto en brotes como en ramas, cor tezas y f lores (Kumazawa *et al*., 2004; Papotti *et al*., 2012). Las más impor tantes fuentes botánicas del propóleos en regiones templadas son el álamo (*Populus* spp.), abedul (*Betula alba*), sauce (*Salix* spp.), pino (*Pinus* spp.), encino (*Quersus* spp.), fresno (*Fraxinus* spp.), entre otros árboles (Far ré *et al*., 2004). No obstante, en las regiones tropicales donde está ausente esta vegetación, las abejas visitan otras plantas como fuente para la producción de propóleos, lo que resulta en diferencias en la composición química (Palomino *et al*., 2010). En regiones mediter ráneas, las fuentes botánicas son los álamos y jaras (*Cistus* spp.), mientras que existen pocos estudios en regiones áridas y semiáridas (Farré *et al*., 2004).

El origen botánico y geográf ico de la zona de recolección está comúnmente ligado a la calidad del propóleos, debido a que la f lora contribuye en algunas de las propiedades físicas como el color, sabor, textura y punto de fusión (Norma Salvadoreña, 2003). Por otro lado, las abejas generalmente son afectadas durante la colecta por insecticidas y otros pesticidas, lo cual puede generar la posible contaminación de la 'carga' y los productos apícolas que posterior mente serán consumidos por los humanos. Por tal razón, los colmenares deben ubicarse lejos de cultivos agrícolas y de las vías de comunicación ter restre, así como de lugares donde se puedan acumular metales pesados (Norma Salvadoreña, 2003; Peña, 2008).

Los diversos productos apícolas, entre ellos el propóleos, son denominados por su origen botánico, es decir, el contenido del polen, el tipo de f lavonoides y otros compuestos presentes, que per miten establecer dicho origen botánico (Kumazawa *et al*., 2004; Valencia *et al*., 2012). En diversos estudios se han establecido diferentes criterios para relacionar propiedades cualitativas y cuantitativas de propóleos de diversas regiones, basándose en el tipo de propóleos, vegetación, estación climática y método de extracción; obteniendo como resultado una amplia variabilidad en los parámetros evaluados (Papotti *et al*., 2012), lo que indica que el origen botánico es muy útil como parámetro de evaluación de la calidad del propóleos. Por lo anterior, su posible empleo en la industria alimenticia genera la necesidad de establecer controles de calidad y nor malización de estos productos (Palomino *et al*., 2010).

Características Fisicoquímicas y organolépticas del Propóleos

El propóleos suele ser un componente aromático debido a su contenido en aceites esenciales y, en función del origen botánico de la resina, época de recolección y edad, dif iere en color (de amarillo claro a castaño oscuro), sabor (amargo, ligeramente picante o insípido) y consistencia, ya que a temperaturas de 45 a 250°C el propóleos es una sustancia suave, f lexible y muy pegajosa, mientras que por debajo de 15°C, se vuelve duro y quebradizo. Normalmente el propóleos es convertido en líquido entre 60 y 70°C, pero para algunas muestras el punto de fusión puede ser de 100°C (Nor ma Salvadoreña, 2003; Nor ma Argentina, 2004; Peña, 2008). Los disolventes más utilizados para la extracción comercial y para el análisis químico son el etanol, propilenglicol, aceite y agua. Muchos de los componentes antioxidantes y antibacterianos presentes en el propóleos son solubles en agua o alcohol, por lo que este tipo de disolventes son ampliamente utilizados al momento de preparar extractos de propóleos con f ines comerciales y de investigación (Bonvehí y Gutiér rez, 2011), lo cual genera diferencias en la composición y actividad del extracto de propóleos obtenido.

Calidad del Propóleos

Las propiedades biológicas que los propóleos poseen pueden ser aprovechadas en la industria alimentaria, cosmética o química. Tales propiedades son dependientes de su composición química, lo que hace necesario establecer un marco nor mativo para evaluar la calidad o características, así como las propiedades y límites de seguridad de este producto (Peña, 2008). Existen antecedentes del uso del propóleos en suplementos y a nivel de investigación, en algunos productos alimenticios. Para que la calidad del propóleos sea considerada aceptable debe cumplir cier tos requisitos, tales como: estar libre de residuos tóxicos; poseer bajo contenido de cera, materia insoluble y cenizas; tener un origen botánico def inir y una actividad biológica comprobada (Tabla I). Actualmente no existe en México una normatividad que in-

TABLA I
CARACTERÍSTICAS GENERALES PARA LA EVALUACIÓN DE LA CALIDAD DEL PROPÓLEOS

Características organolépticas	Consideraciones
Aroma	Inodoro, resinoso suave, aromático o balsámico
Color	Amarillo, café, verde, rojo y gris, y sus tonalidades
Sabor	Picante, dulce, amargo e insípido
Consistencia a temperatura ambiente	Muy blanda, blanda, dura, poco dura, pegajosa y porosa
Aspecto	Homogéneo o heterogéneo
Carácterísticas físicas y químicas	
Extracto seco	Mínimo 10%
índice de oxidación	Máximo 22 segundos
Compuestos fenólicos (mg AG/ml)	Mínimo 0.25-5%
Flavonoides	Mínimo 0.25-0.5%
Espectrograma UV-VIS	Máximo de absorción entre 270 y 315 nm
Metales pesados: plomo y arsénico	Máximo $2mg \cdot kg^{-1}$ y $1mg \cdot kg^{-1}$, respectivamente
Residuos de plaguicidas y antibióticos	Ausente
Humedad	Máximo 8%
Cenizas	Máximo 5%
Cera	Máximo 30%
Impurezas mecánicas	25-30%
índice de yodo	Mínimo 35%
Solubilidad en etanol	30-35%
Características microbiológicas	
Bacterias mesófilas (UFC/g)	<10,000
Coliformes fecales (UFC/g)	0
Coliformes totales (UFC/g)	<100
Staphylococcus aureus (UFC/g)	100
Hongos(UFC/g)	1-1000

Fuentes: Norma Rusa (1977), Norma Cubana (1994), Norma Salvadoreña (2003) y Norma Argentina (2004).

dique los requerimientos necesarios para establecer el uso del propóleos en alimentos. Sin embargo, a nivel inter nacional existen nor mas, tales como la Nor ma Rusa (1977), la Nor ma Cubana (1994), la Nor ma Salvadoreña (2003) y la Nor ma Argentina, 2004, las cuales establecen la identidad y los requisitos mínimos de calidad que debe cumplir el propóleos cr udo para su comercialización, con la f inalidad de que los productores adopten medidas destinadas a proteger la salud de la población. Actualmente, en las mismas nor mas se ha establecido que se autoriza el uso del propóleos como ingrediente únicamente en productos como caramelos, mieles, y extractos de propóleos, ya sea en solución hidroalcohólica de etanol o propilenglicol, además de cier tos suplementos dietéticos.

Composición Química

La composición química del propóleos es muy variable y compleja. Depende, como se mencionó anterior mente, de la f lora local cercana al sitio donde se encuentra la colmena. Sus principales componentes son resinas y bálsamos (50-55%), ceras (25-35%), aceites volátiles (10%), polen (5%), minerales y sustancias orgánicas (5%). Entre estas últimas se han detectado ácidos orgánicos, ácidos fenólicos (Nagai *et al*., 2003; Papotti *et al*., 2012), aldehídos aromáticos, cumarinas, compuestos fenólicos como f lavonoides (f lavonas, f lavonoles, f lavanonas, f lavononoles) y minerales (Al, Ag, Ba, B, Cr, Co, Cu, Sn, Fe, Mg, Mn, Mb, Ni, Pb, Se, Si, Sr, Ti, V y Zn) (Nagai *et al*., 2003; Kumazawa *et al*., 2004). Pueden estar presentes las vitaminas A, B1, B2, B3 y B6 (Kumazawa *et al*., 2004; Chaillou y Nazareno, 2009).

El gr upo de compuestos con actividad biológica más comúnmente evaluados en extractos de propóleos son los conocidos como compuestos fenólicos, y la mayoría de éstos se presentan en for ma de f lavonoides (Tabla II), cuya concentración y actividad biológica dependerán del tipo de planta en la cual se llevó a cabo la recolección (Bedascar rasbure *et al*., 2004; Yang *et al*., 2011). Así, la presencia de ocho f lavonoides, incluyendo r utina, miricetina, quercetina, kaempferol, apigenina, pinocembrina, crisina y galangina, puede ser usada como marcador para diferenciar al propóleos de otros productos de la colmena. El contenido de estos ocho flavonoides ha sido extensamente usado como parámetro de la calidad del propóleos (Viuda-Martos *et al*., 2008).

El efecto de los propóleos sobre una gran variedad de micror ganismos (bacterias, hongos, vir us y levaduras) ha sido ampliamente comprobado y se ha demuestrado que el efecto es dependiente de la composición química (Kumar *et al*., 2008; Viuda-Martos *et al*., 2008). Además de las propiedades antimicrobianas del propóleos, su actividad antioxidante también depende de la composición, pudiendo estas propiedades ser aprovechadas para alargar la vida de anaquel de algunos productos alimenticios, ya que los principales factores que afectan la vida útil de un alimento son las reacciones de oxidación de lípidos y la contaminación por bacterias y hongos patógenos (Peña, 2008; Yue-Wen *et al*., 2008).

Actividad Antioxidante

Uno de los mayores cambios que ocur ren durante el proceso, distribución y preparación de los alimentos es la oxidación de lípidos, la que provoca cambios en el sistema alimenticio afectando su calidad nutricional, seguridad, color, olor, sabor y textura, con resultado f inal de rechazo por los consumidores. Por ello, en la industria alimentaria se utilizan antioxidantes sintéticos para dar estabilidad al alimento, siendo el butilhidroxianisol (BHA), butilhidroxitolueno (BHT) y terbutilhidroxiquinona (TBHQ) los más comúnmente empleados para prevenir el deterioro oxidativo. Sin embargo, se ha encontrado que éstos pueden ser promotores de tumores y cáncer (Huang *et al*., 2011).

Actualmente la industria agroalimentaria enfrenta continuamente problemas derivados de la falta de estabilidad de los alimentos, siendo la principal causa la oxidación de los mismos; así el uso de antioxidantes naturales puede resultar una alter nativa (Fer nández-López *et al*., 2005): Tal es el caso del propóleos, ya que debido a su composición química es una fuente natural de antioxidantes. Las propiedades antioxidantes del propóleos se deben a la actividad antiradical y al efecto inhibidor sobre iones metálicos que éstos poseen (Gülçin *et al*., 2010). Mendes da Silva *et al*. (2006) demostraron que los extractos etanólicos de propóleos brasileños probados *in vitro* poseen alta capacidad antioxidante, y ésta se cor relacionó con su contenido total de f lavonoides. Por otro lado, algunos estudios indican que cier tos f lavonoides poseen acciones prooxidante; sin embar-

TABLA II
PRINCIPALES COMPUESTOS DEL PROPÓLEOS CON ACTIVIDAD BIOLÓGICA

Bioactividad	Compuesto	Denominación IUPAC	Número CAS	Referencias
Antioxidante	Acacetina Ácido	5,7-dihidroxi-2-(4-metoxifenil) croman-4-uno	480-44-4	Bedascarrasbure *et al*., 2004; Velazquez *et al*., 2007; Yang *et al*., 2011; Valencia *et al*., 2012.
	cafeico Ácido	(E)-3-(3,4-dihidroxifenol)- ácido 2-propenoico	331-39-5	
	cinámico Ácido	(E)-3-fenil-ácido propil 2-enoico	140-10-3	
	ferúlico Ácido	(E)-3-(4-hidroxi-3-metoxifenil) ácido propil-2-enoico	537-98-4	
	sinapínico Ácido	(E)-3-(4-hidroxi-3,5-dimetoxifenil) ácido propil-2-enoico	530-59-6	
	p-cumárico	(E)-3-(4-hidroxifenil)- ácido 2-propenoico	501-98-4	
	Apigenina	5,7-dihidroxi-2-(4-hidroxifenil)-croman-4-uno	520-36-5	
	Artepillin C	(E)-3-[4-hidroxi-3,5-bis(3-metil-2-butenil) fenil] ácido propenoico	72944-19-5	
	Éster fenetílico del ácido cafeico (CAPE)	(E)-3-(3,4-dihidroxifenil)- ácido 2-propenoico, 2-éster fenetílico	104594-70-9	
	Galangina	3,5,7-trihidroxi-2-fenilcroman-4-uno	548-83-4	
	Kaempferol	3,5,7-trihidroxi-2-(4-hidroxifenil)-croman-4-uno	520-18-3	
	Pinocembrina	(2S)-5,7-dihidroxi-2-fenil-2,3-dihidrocroman-4-uno	480-39-7	
	Quercetina	2-(3,4-dihidroxifenil)-3,5,7-trihidroxicroman-4-uno	117-39-5	
Antimicrobiana	Ácido cafeico	(E)-3-(3,4-dihidroxifenol)- ácido 2-propenoico	331-39-5	Takaisi and Schilcher, 1994; Mirzoeva *et al*., 1997; Velazquez *et al*., 2007; Ahn *et al*., 2009.
	Ácido p-cumárico	(E)-3-(4-hidroxifenil)- ácido 2-propenoico	501-98-4	
	Crisina	5,7-dihidroxi-2-fenilcroman-4-uno	480-40-0	
	Éster fenetílico del ácido cafeico (CAPE)	(E)-3-(3,4-dihidroxifenil)- ácido2-propenoico, 2-éster fenetil	104594-70-9	
	Galangina	3,5,7-trihidroxi-2-fenilcroman-4-uno	548-83-4	
	Naringenina	(2S)-5,7-dihidroxi-2-(4-hidroxifenil)-2,3-dihidrochroman-4-uno	10236-47-29	
	Pinobanksina	(2R,3R)-3,5,7-trihidroxi-2-fenil-2,3-dihidrocroman-4-uno	548-82-3	
	Pinobanksina-3-acetato	[(2R,3R)-5,7-dihidroxi-4-oxo-2-fenil-2,3-dihidrocroman-3-yl] acetato	52117-69-8	
	Pinocembrina	(2S)-5,7-dihidroxi-2-fenil-2,3-dihidrocroman-4-uno	480-39-7	
	Quercetina	2-(3,4-dihidroxifenil)-3,5,7-trihidroxicroman-4-uno	117-39-5	
Antifúngica	Ácido cafeico	(E)-3-(3,4-dihidroxifenil- ácido 2-propenoico	331-39-5	Bedascarrasbure *et al*., 2004; Chaillou y Nazareno, 2009.
	Ácido ferúlico	(E)-3-(4-hidroxi-3-metoxifenil) ácido propil-2-enoico	537-98-4	
	Ácido p-cumárico	(E)-3-(4-hidroxifenil)- ácido 2-propenoico	501-98-4	
	Galangina	3,5,7-trihidroxi-2-fenilcroman-4-uno	548-83-4	
	Pinocembrina	(2S)-5,7-dihidroxi-2-fenil-2,3-dihidrocroman-4-uno	480-39-7	

go, este efecto sólo se observa cuando las concentraciones utilizadas son altas (Mar tinez-Flores *et al*., 2002). Valencia *et al*. (2012), evaluaron la capacidad de los propóleos sonorenses (México) para atrapar radicales libres (DPPH), y co- r relacionaron esta actividad con el alto contenido de compuestos fenólicos y flavonoides.

Han y Park (2002) evaluaron la acumulación de sustancias reactivas al ácido tiobarbitúrico (TBARS) en salchichas de cerdo tratadas con sorbato de potasio y con extractos de propóleos secos, etanólicos y acuosos, las cuales se almacenaron a 5, 10 y 20°C durante cuatro semanas, repor tando un incremento en los valores de TBARS desde el inicio del almacenamiento en todos los tratamientos, aunque los valores más bajos de TBARS fueron para las salchichas tratadas con los extractos de propóleos. Por otro lado, In-suk *et al*. (2002) evaluaron el efecto antioxidante del propóleos al 1, 3 y 5% para preser var salchichas de cerdo almacenadas a 20°C, durante siete días. De igual manera, se encontraron valores bajos de TBARS en las salchichas tratadas con propóleos durante todos los días de muestreo. Ambas investigaciones demuestran que hubo una reducción en la velocidad de oxidación y un mayor tiempo de conservación de las salchichas de cerdo. Sánchez-Escalante *et al*. (2009), evaluaron el efecto antioxidante de propóleos producidos en el noroeste de México en hamburguesas de bovino almacenadas a 2°C, sin iluminación. Ambos propóleos conser varon el color característico de la car ne fresca durante ocho días y disminuyeron la for mación de metamioglobina, TBARS y dienos conjugados. Los resultados sugieren que el propóleos es un antioxidante natural potencial, el cual puede ser usado en productos cárnicos frescos para extender su vida de anaquel.

Actividad Antimicrobiana

Las propiedades antimicrobianas del propóleos han sido estudiadas desde finales de la década de los 40. En ensayos *in vitro* se ha demostrado que los extractos de propóleos son activos frente a numerosos microorganismos, siendo más eficaces en un g ran número de bacter ias g ram positivas, pero con limitada actividad sobre gram negativas (Marcucci *et al*., 2001; Peña, 2008). Choi *et al*. (2006) est udiaron la actividad antimicrobiana de los ext ractos de propóleos de alg unas regiones de Corea y encont raron que éstos tenían alta actividad sobre *S. aureus, B. subtilis, S. t yphimurium y C. albicans*. ot ras investigaciones mencionan la reducción *in vitro* en el número de microorganismos (*S. aureus, E. faecalis, B. subtilis, S. t yphimurium y C. albicans*), at r ibuyendo esta actividad al contenido de compuestos fenólicos como los f lavonoides (Velázquez *et al*. 2007).

Respecto a estudios realizados para probar la actividad antimicrobiana en alimentos, In-Suk *et al*. (2002) evaluaron el efecto de extractos etanólicos de propóleos (EEP) en salchichas de cerdo, sobre la cuenta total de mesóf ilos y colifor mes. Las salchichas fueron almacenadas a 20°C durante siete días, encontrándose una reducción muy impor tante de la carga bacteriana a par tir del tercer día de almacenamiento. En otro estudio, Sagdic *et al*. (2007) evaluaron el efecto de extractos acuosos de propóleos sobre el crecimiento de *Escherichia coli* y *E. coli* o157:H7 en jugo de manzana; los autores encontraron una importante reduc-

TABLA III
PROPIEDADES BIOLÓGICAS DEL PROPÓLEOS DE INTERÉS PARA LA INDUSTRIA ALIMENTARIA

Bioactividad	Comentarios	Actividad	Referencias	Posible aplicación en alimentos
Antioxidante	Quelación de radicales y iones metálicos; poder reductor; inhibición de peróxido de hidrógeno (H_2O_2), y radicales superóxido (o_2), hidroxilo (Ho), alcoxilo (Ro^-), peroxilo (Roo^-).	*In vitro*	Choi *et al*., 2006; Velazquez *et al*., 2007; Bonvehí *et al*., 2011; Yang *et al*., 2011; Valencia *et al*., 2012.	-Productos cárnicos (res, pollo, cerdo, pescado o mariscos). -Aceites vegetales.
Antimicrobiana	*Bacillus subtilis, Costridium perfringens, Escherichea coli, Escherichea coli* o157:H7; *Listeria monocytogenes, Pseudomona aeruginosa, Salmonella typhi, Salmonella typhimuriu, Staphyloccous aureus, Vibrio cholerae* y *Vibrio parahaemolyticus*	*In vitro*	Marcucci *et al*., 2001; Kosalec *et al*., 2005; Choi *et al*., 2006; Sagdic *et al*., 2007; Velazquez *et al*., 2007; Kumar *et al*., 2008.	-Productos lácteos sin pasteurizar. -Productos cárnicos. -Jugo de frutas. -Alimentos refrigerados, listos para consumir.
	Campylobacter jejuni, Clostridium botulinum, Shigella y *Yersinia enterocolitica*	-	No existen estudios	-Productos lácteos, cárnicos y alimentos enlatados.
Antifúngica	*Absidia corymbifera,Aspergillus fumigatus, Aspergillus sulphureus, Candida albicans, Candida krusei, Candida tropicalis, Candida glabrata, C. kefyr, C. parapsilosis, C. famata, C. glabrata, C. pelliculosa, Colletotrichum gloeosporioides, Cryptococcus neoformans, Trichophyton mentagrophytes, Phytophthora capsisi* y *Phytophthora infestans, Phytophthora parasítica* y *P. ohmeri*	*In vitro*	Choi *et al*., 2006; Mercan *et al*., 2006; Koc *et al*., 2007; Kosalec *et al*., 2005; Kumar *et al*., 2008; Meneses *et al*., 2009; Pineda *et al*., 2010.	-Frutas: Aguacate (*Persea americana*), papaya (*Carica papaya*); maracuya (*Passiflora edulis*); Mango (*Mangifera indica L.*) Jugos de fruta: manzana, mandarina, naranja, uva blanca.

ción en el número de estos microorganismos gram negativos por efecto de la presencia de propóleos, lo cual indica que éste podría ser utilizado como un agente bactericida contra *E. coli* y *E. coli* o157:H7 en jugo de manzana, incrementando la vida útil, sobre todo cuando se almacena a temperatura ambiente, y con un gran potencial como alternativa natural en la conservación de los alimentos, siempre y cuando sea organolépticamente aceptable.

La actividad antibacteriana de los propóleos varía dependiendo de la composición química, dosis y solvente de extracción o preparación (especialmente extractos etanólicos). Vargas-Sánchez *et al*. (2011) evaluaron las propiedades antioxidantes y antimicrobianas de propóleos de tres diferentes fuentes: propóleos comercial 1 (PC1), propóleos comercial 2 (PC2) y un tercer extracto no comercial obtenido de la región de Pueblo de Álamos, Sonora, México (PA P). En este estudio se evaluó *in vitro* la concentración mínima inhibitoria de los extractos (300, 60, 30 y 15µg·ml^{-1}) frente a *S. aureus, L. monocytogenes, Salmonella* spp, y *E. coli* o157:H7. Además, se trataron hamburguesas de carne bovina con extractos de propóleos (2%), las cuales fueron almacenadas a 2°C, sin iluminación durante dos semanas. Los extractos de propóleos presentaron alta actividad frente bacterias gram-positivas (*S. aureus* y *L. monocytogenes*) y disminuyeron la población (UFC/g) de microorganismos mesófilos y psicrótrofos aerobios. Estos resultados muestran que el propóleos es una alternativa prometedora a los antibacterianos existentes y puede ser también empleado para extender la vida de anaquel de la carne fresca.

Actividad Antifúngica

Al igual que el deterioro bacteriano, la contaminación por hongos constituye un serio problema para la industria alimentaria y esta puede ocurrir durante el procesamiento, así como en el manejo final del producto. Por ello se han empleado conservadores químicos en los alimentos (benzoato de sodio, sorbato de potasio y sus mezclas), para evitar este tipo de deterioro (Koc *et al*., 2007; Meneses *et al*., 2009). En los últimos años la atención se ha centrado en el uso del propóleos como un suplemento alimenticio adecuado para los consumidores en los países desarrollados (Meneses *et al*., 2009) y como una alternativa al uso de productos químicos para el control de hongos, los que ocasionan diversos problemas ecológicos. Ante esta situación, el propóleos se presenta como una alternativa de control que puede actuar de forma equilibrada con el ambiente debido a su origen natural (Principal *et al*., 2002; Meneses *et al*., 2009).

Pineda *et al*. (2010) evaluaron el efecto antifúngico del propóleos en etanol (0, 15, 20 y 30%) sobre aislados de *Colletotrichum gloeosporioides* provenientes de aguacate (*Persea americana*), papaya (*Carica papaya*) y maracuyá *(Passiflora edulis).* Los resultados mostraron el efecto supresor del propóleos sobre el crecimiento micelial de *C. gloeosporioides*, lo cual fue atribuido a la presencia de compuestos flavonoides en el extracto. *C. gloeosporioides* es un hongo patógeno causante de la antracnosis, patología que acelera la pudrición de muchos frutos, reduciendo su valor comercial. La enfermedad se expresa principalmente cuando los frutos comienzan a madurar, causando manchas necróticas depresivas sobre la superficie, sobre todo en condiciones de mal manejo durante el transporte y almacenamiento del producto (Meneses *et al*., 2009). Además de las lesiones externas que causa, el hongo penetra el interior del fruto, alterando la pulpa y causando pudrición de tejidos internos.

Por otra parte, Pineda *et al*. (2010) demostraron el efecto de inhibición del propóleos sobre el crecimiento micelial de fitopatógenos, lo que fue correlacionado a la presencia de compuestos flavonoides en el extracto. Koc *et al*. (2007) determinaron

la efectividad del propóleos *in vitro* sobre aislados de jugo de f r utas, encont rando que el propóleos posee actividad antif úngica sig nif icativa sobre las levaduras aisladas, y concluyeron que el propóleos es merecedor de un est udio más prof undo para ser designado como un conser vador nat ural para alimentos propensos al deter ioro por hongos.

El est udio de la acción antif úngica del propóleos sobre f itopatógenos per mitirá el cont rol de éstos con est rategias no contaminantes, lo cual sig nif icará un avance hacia la ag r icult ura sostenible. Así, las propiedades antioxidantes, antibacter ianas y antif úngicas del propóleos ha sido bien documentada por diversos autores a escala mundial (Tabla III); sin embargo, son pocos los t rabajos publicados sobre su uso en productos alimenticios.

Mecanismo de Acción del Propóleos

Mirzoeva *et al.* (1997) demost raron que alg unos de los constit uyentes común mente encont rados en los propóleos, pr incipalmente quercetina y nar ingenina, provocan un incremento en la per meabilidad y una reducción en el potencial de la membrana bacter iana, lo que cont r ibuye a disminuir la resistencia de las bacter ias a agentes antibacterianos. Estos f lavonoides también in hibieron la motilidad bacter iana, factor impor tante en la vir ulencia de estos microorganismos. Por ot ra par te, Cush nie y Lamb (2005) repor taron que la galangina incrementa la pérdida de potasio en *Staphyloccocus aureus*, deg radando la membrana citoplasmática de las bacter ias por lisis osmótica, demost rándose la efectiva actividad antimicrobiana de este pro - ducto. Act ualmente no están completamente est udiados todos los mecanismos por los cuales el propóleos puede in hibir la presencia de cier tos patógenos; sin embargo, alg unos de los componentes encont rados en los propóleos, como ácidos aromáticos y ésteres, compuestos cinámicos y f lavónicos, alteran las membranas celulares, in hiben la A R N polimerasa y reducen la motilidad bacter iana, lo cual cont r ibuye a su acción y al sinergismo obser vado con alg unos antibióticos (Takaisi-Kik uni y Schilcher, 1994; Mirzoeva *et al*., 1997).

Respecto a la actividad antioxidante del propóleos, Yang *et al.* (2011) encont raron que éste posee la habilidad de at rapar radicales libres, lo que demuest ra que éste es uno de los mecanismos mediante el cual el propóleos ejerce su potencial antioxidante. En ot ra investigación realizada por Gülçin *et al.* (2010) se demost ró que el propóleos posee la habilidad para quelar los iones Fe^{3+}, Cu^{2+} y Fe^{2+}, atrapar radicales DPPH y $ABTS^{+}$ e inhibir la peroxidación lipídica.

Conclusiones

A pesar de los atributos biológicos que posee este producto apícola, al propóleos no se le ha dado una aplicación como conser vador potencial en tecnología de alimentos. La infor mación presentada en esta revisión revela que las propiedades antioxidantes, antimicrobianas y antifúngicas presentes en el propóleos pueden brindar la posibilidad a la industria alimentaria de una gran variedad de aplicaciones, utilizándolo como un conser vador natural que per mita retardar la descomposición de alimentos durante su almacenamiento, con la ventaja de que los compuestos presentes pueden ser benef iciosos para la salud humana. Por ello es necesario realizar estudios que profundicen acerca de los posibles efectos que a largo plazo se deriven de su consumo, y sobre el posible efecto en las propiedades de los alimentos procesados.

REFERENCIAS

Bedascar rasbure E, Maldonado L, Alvarez A, Rodríguez E (2004) Contenido de fenoles y f lavonoides del propóleos Argentino. *Acta Farm. Bonaerense. 23*: 369-372.

Bonvehí JS, Gutiér rez AL (2011) Antioxidant activity and total phenolics of propolis from the basque countr y (Nor theaster n Spain). *J. Am. Oil Chem. Soc. 88*: 1387-1395.

Brook man P (1991) Antioxidants and consumer acceptance. *Food Tech. 26*: 24-28.

Chaillou LL, Nazareno MA (2009) Bioactivity of propolis for m Santiago del Estero, Argentina, related to their chemical composition. *Food Sci. Technol. 42*: 1422-1427.

Choi YM, Noh Do, Cho SY, Suh HJ, Kim K M, Kim J M (2006) Antioxidant and antimicrobial activities of propolis from several regions of Korea. *Food Sci. Technol. 39*: 756-761.

Cushnie TPT, Lamb AJ (2005) Detection of galangin-induced cytoplasmic membrane damage in Staphylococcus aureus by measuring potassium loss. *J Ethnopharmacol. 101*: 243-248.

Farré R, Frasquet I, Sánchez A (2004) El propólis y la salud. *Ars Pharm. 45*: 23-43.

Fer nández-López J, Zhi N, Aleson-Carbonell L, Pérez-Alvarez JA, Kuri V (2005) Antioxidant and antibacterial activities of nat ural extracts: application in beef meatballs. *Meat Sci. 69*: 371-380.

Gregoris E, Fabris S, Ber telle M, Grassato L, Stevanato R (2011) Propolis as potential cosmeceutical sunscreen agent for its combined photoprotective and antioxidant properties. *Int J. Pharm. 405*: 97-101.

Gülçin I, Bursal E, Sehitoglu MH, Bilsel M, Görden AC (2010) Poly phenols contents and antioxidant activity of lyophilized aqueous extract of propolis from Erzur um, Turkey. *Food Chem Toxicol. 48*: 2227-2238.

Han SK, Park HK (2002) Accumulation of thiobarbit uric acid-reactive substances in cured pork sausages treated with propolis extracts. *J. Sci. Food Agric. 82*: 1487-1489.

Huang B, He J, Ban X, Zeng H, Yao X, Wang Y (2011) Antioxidant activity of bovine and porcine meat treated with extracts from edible lot us (*Nelumbo nucifera*) rhizoma knot and leaf. *Meat Sci. 87*: 46-53.

In-Suk o, Dong-Hwan o, Young-Sook C, Kap-Suk K, Mi-Yae S, Kwon-Il S (2002) Effects of ethanol extract of propolis (EEP) on the storage of sausage. *Kor. Soc. Food Sci Nutr. 7*: 35-39.

Koc AN, Silici S, Mutlu-Sariguzel F, Sagdic o (2007) Antif ungal activity of propolis in four different fr uit juices. *Food Technol Biotechnol. 45*: 57-61.

Kosalec I, Pepeljnjak S, Bak maz M, Vladimir-K nezevic S (2005) Flavonoid analysis and antimicrobial activity of commercially available propolis products. *Acta Pharm. 55*: 423-430.

Kumar N, Mueen-Ahmad K K, Dang R, Husain A (2008) Antioxidant and antimicrobial activity of propolis from Tamil Nadu zone. *J. Med. Plants Res. 2*: 361-364.

Kumazawa S, Hamasaka T, Nakayama T (2004) Antioxidant activity of propolis of various geographic origins. *Food Chem. 84*: 329-339.

Manrique AJ, Santana WC (2008) Flavonoides, actividades antibacteriana y antioxidante de propóleos de abejas sin aguijón, *Melipona quadrifasciata*, *Melipona compressipes*, *Tetragonisca angustula* y *Nannotrigona* sp. de Brasil y Venezuela. *Zootecnia Trop. 26*: 157-166.

Marcucci MC, Fer reres F, García-Viguera C, Ban kova VS, De Castro SL, Dantas AP (2001) Phenolic compounds from Brazilian propolis with phar macological activities. *J Ethnopharmacol. 74*: 105-112.

Mar tínez-Flórez S, González-Gallego J, Culebras J M, Tuñón MJ (2002) Los f lavonoides: propiedades y acciones antioxidantes. Revisión. *Nutr. Hosp. 8*: 271-278.

Mendes da Silva J F, De Souza MC, Ramalho MS, Ribeiro AM, Nova VFV (2006) Co- r relation analysis between phenolic levels of Brazilian propolis extracts and their antimicrobial and antioxidant activities. *Food Chem. 99*: 431-435.

Meneses EA, Durango DL, García CM (2009) Antif ungal activity against posthar vest f ungi by extracts from Colombian propolis. *Quim. Nova 32*: 2011-2017.

Mirzoeva oK, Grisjanin R N, Calder PC (1997) Antimicrobial action of propolis and some of its components: the effects on growth, membrane potential and motility of bacteria. *Microbiol. Res. 152*: 239-246.

Nagai T, Inoue R, Inoue H, Suzuki N (2003) Preparation and antioxidant properties of water extract of propolis. *Food Chem. 80*: 29-33.

Norma Argentina (2004) *Propóleos en Buto*. Norma IRAM-INTA 15925-1. [illegible]

gentino de Normalización. Buenos Aires, Argentina.

Nor ma Cubana (1994) *Propóleos Materia Prima. Especif icaciones.* Nor ma Ramal Cubana. Apicult ura NR AG-1135-94. Ministerio de Agricultura. La Habana, Cuba.

Nor ma Rusa (1977) *Propóleos. Métodos analíticos para el control de su calidad*. Nor ma Ramal Rusa RST-RSFSR-317-77.

Nor ma Salvadoreña (2003) *Calidad del Propólero Crudo.* Nor ma NSo 65.19.02:03. Diario of icial. Tomo 360. San Salvador, El Salvador.

Palomino GR, Mar tínez GJ, García PM, Gil GJ, Durango R D (2010) Caracterización f isicoquímica y actividad antimicrobiana del propóleos en el Municipio de La Unión (Antioquia, Colombia). *Rev. Fac. Nac. Agric. Medellín 63*: 5373-5383.

Papotti G, Ber telli D, Bor tolotti L, Plessi M (2012) Chemical and f unctional characterization of Italian propolis obtained by different har vesting methods. *J. Agric Food Chem. 60*: 2852-2862.

Peña RC (2008) Literat ure review: Propolis standardization: a chemical and biological review. *Cienc. Inv. Agr. 35*: 11-20.

Pineda J, Pincipal J, Barrios C, Milla D, Solano Y, Gil E (2010) Propiedad fungistática in vitro de propóleos sobre tres aislamientos de *Colletotrichum gloeosporioides. Zootecn. Trop. 28*: 83-91.

Principal J, Her nández I, D'Aubeter re R, Rodríguez JG (2002) Ef icacia del propóleos en el control de la helmintiasis de ovinos nat uralmente infestados. *Rev. Cient. Fac. Cs. Vet. LUZ 8*: 604-607.

Sagdic o, Silici S, Yetim H (2007) Fate of *Escherichia coli* and *E. coli* o157:H7 in apple juice treated with propolis extract. *Ann. Microbiol. 57*: 345-348.

Sánchez-Escalante A, Vargas-Sánchez R D, Valenzuela M, De la Rosa MA, Tor rescano G, Camou J P (2009) Evaluation of antioxidant activity of propolis produced in northwest of Mexico for fresh beef patties. *Annu. Meet. Institute of Food Technologists.* Anaheim, CA, EEUU.

Takaisi-Kikuni NB, Schilcher H (1994) Electron microscopic and microcalorimetric investigations of the possible mechanism of the antibacterial action of a def ined propolis provenance. *Planta Medica. 60*: 222-227.

Valencia D, Alday E, Robles-Zepeda R, Garibay-Escobar A, Galvez-Ruiz JC, Salas-Reyes M., Jiménez-Estrada M, Velazquez-Contreras E, Her nandez J, Velazquez C (2012) Seasonal effect on chemical composition and biological activities of Sonoran propolis. *Food Chem. 131*: 645-651.

Vargas-Sánchez R, Javier-Saiz E, Tor rescano-Ur r utia G, Acedo E, Car vajal E, González-Córdova A, Vallejo-Galland B, Tor res-Llañez M, Sánchez-Escalante (2011) Antioxidant and antimicrobial proper ties of commercial propolis in beef patties. *Annu. Meet. Institute of Food Technologists.* New orleans, LA, EEUU.

Velazquez C, Navarro M, Acosta A, Angulo A, Dominguez Z, Robles R, Robles-Zepeda R, Lugo E, Goycoolea FM, Velazquez EF, Astiazaran H, Hernández J (2007) Antibacterial and free-radical scavenging activities of Sonoran propolis. *J. Appl. Microbiol. 103*: 1747-1756.

Viuda-Mar tos M, Ruiz-Navajas Y, Fer nández-López J, Pérez-Álvarez JA (2008) Functional proper ties of honey, propolis, and royal jelly. *JFS. R: Conc. Rev. Hypoth. Food Sc. 73*: 117-124.

Yang H, Dong Y, Du H, Shi H, Peng Y, Li X (2011) Antioxidant compounds from propolis collected in Anhui, China. *Molecules 16*: 3444-3455.

Yue-Wen C, Shiao-Wen W, Kai-Kuang H, Shih-Bin L, Chung-Yang H, Chia-Nan C (2008) Characterisation of Taiwanese propolis collected from different locations and seasons. *J. Sci. Food Agric. 88*: 412-419.

PROPOLIS: A POTENCIAL PRESERVATION AGENT FOR THE FOOD INDUSTRY

Rey David Vargas-Sánchez, Gastón R. Torrescano-Urrutia and Armida Sánchez-Escalante

SUMMARY

Propolis is a beehive product made up of resins that bees collect from certain plant species. Diverse studies show that they have, among others, antioxidant, antimicrobial and antifungal properties, which are dependent of their botanical origin, chemical composition, seasons, extraction method, age and collection area. Being a natural product, propolis is usually classed as a GRAS (Generally recognized as safe) product. In some studies, propolis extracts have been shown to have effects upon certain bacteria and fungi, as well as upon other food pathogens, besides their capacity to prevent or retard oxidation reactions, which turns them into potentially attractive natural products to be used as food preservers in substitution of synthetic agents. It is concluded that propolis can be incorporated into food thanks to their diverse biological properties.

O PRÓPOLIS: CONSERVADOR POTENCIAL PARA A INDÚSTRIA ALIMENTÍCIA

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RESUMO

O própolis é um produto da colmeia formado por resinas que as abelhas coletam de certas espécies de plantas. Diversos estudos demonstram que possui propriedades antioxidantes, antimicrobianas e antifúngicas, entre outras, as quais dependem de sua origem botânica, composição química, estação climática, método de extração, idade e área geográfica de coleta. O própolis, por ser um produto natural recebe a denominação GRAS (Geralmente reconhecido como seguro). Em alguns estudos tem sido demonstrado o efeito dos extratos de própolis sobre certas bactérias e fungos, assim como patogênicos de interesse alimentar, além da capacidade que têm para prevenir ou retardar reações de oxidação, o qual os converte em produtos naturais potencialmente atrativos para ser utilizado como conservador alimentar, em substituição dos aditivos sintéticos. Conclui-se que os extratos de própolis podem ser incorporados em matrizes alimentícias devido às diversas propriedades biológicas que apresentam.

CAPÍTULO II

Effect of propolis extract in the oxidative stability of raw bovine and porcine patties extract during chill storage.

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Artículo en preparación para la revista

Meat Science

Effect of propolis extract in the oxidative stability of raw bovine and porcine patties extract during chill storage

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ABSTRACT

Effect of propolis extract (PE), butylated hydroxy toluene (BHT) and ascorbic acid (Asc ac) at 2, 0.2 and 0.15% (w/w), respectively, as inhibitors of lipid (Lox) and protein oxidation (Pox), color deterioration and antioxidant stabilizer of raw bovine and porcine patties during chilled storage (2 °C/9 days/under darkness) was investigated. Bovine and porcine patties were evaluated for pH, lipid oxidation (TBARS), protein oxidation (Carbonyls), color (L*, a*, b*, C* and H*), metmyoglobin formation (MetMb%), total phenolic content (TPC), reducing power (RP) and radical-scavenging activity (DPPH). The antioxidants used were characterized by their *in vitro* antioxidant activity. Results indicated that PE are rich in phenolic content and their incorporation in bovine and porcine patties significantly reduce (P<0.05) lipid and protein oxidation (TBARS-80.0 and 88.7% inhibition; Pox-47.3 and 30.6% inhibition, respectively), as well as loss color and increase the oxidative stability of patties during chill storage.

Keywords: Propolis extract, Lipid oxidation, Protein oxidation, Color stability, Meat.

1. Introduction

Beef and Pork meat is a major source of high quality dietary nutrients for human metabolic processes such as proteins and fat content (Kauffman, 2001; Cabrera, & Saadoun, 2014). Fat provides indispensable dietary energy and essential nutrients such as essential fatty acids and fat-soluble vitamins. The lipid content of meat contributes to its cooking characteristics, palatability and overall organoleptic properties (Wood et al., 2008). Additionally, meat protein distinguishes due its richness in all the essential amino acids (Williams, 2007). However, beef and pork meat also contributes to the intake of fat, saturated fatty acids, cholesterol, and other protein derivates substances that, in inappropriate amounts, may result in negative physiologically effects and quality loss (Xiong, 2011; Toldrá, & Reig, 2011).

Oxidative degradation of polyunsaturated fatty acids (PUFAs) during storage it considere the main causes of quality deterioration in meat and meat products, causing changes in texture, color, smell, taste and loss of nutrients (Löliger & Wille, 1993). In relation to protein oxidation, the nature of the oxidation products formed is highly dependent on the amino acids involved, how the oxidation process start, may decrease eating quality by reducing tenderness and juiciness, enhancing flavor deterioration and discoloration of meat, all of which are the major quality factors affecting consumers' acceptance of meat (Lund, Heinonen, Baron, & Estevez, 2011). Although synthetic additives have been widely used in the meat industry to inhibit both, process of lipid and protein oxidation (Lox and Pox). However, the use of synthetic antioxidants has shown potential health risks, promoting strict regulations for their use in foods (Brookman, 1991; Löliger & Wille, 1993). Natural antioxidants, such as honey, royal jelly and propolis have shown to decrease oxidation as effectively as synthetic antioxidant. Therefore, it is important replaces synthetic antioxidants with natural ingredients (Faustman, Sun, Mancini, & Suman, 2010).

Propolis is a substance of complex composition and viscous consistency, which bees made from resinous material from different plants. Many studies have demonstrated the excellent biological principles of propolis extract such as antimicrobial, antifungal, anticancer, antiinflammatory and antioxidant, among others, which vary depending on its botanical source, season of collection and phenolic

composition (Farré, 2004). In particular, phenolic constituents of propolis extracts have received much interest due its remarkable ability to capture free radicals (Valencia et al., 2012). Several methods such as total phenolic content, reducing power and radical-scavenging activity, among others, have been used to determine antioxidant properties of food systems such as vegetables, fruits, and spices (Geckil et al., 2005; Tan, & Lim, 2015). Also, measurement of antioxidant activity in muscles from different species have increased in recent years (Huang et al., 2011; Mielnik, Rzeszutek, Triumf, & Egelandsdal, 2011); therefore, it is necessary to determine the effect of propolis extracts application in diverse meat types, including bovine and porcine muscle, as an antioxidant and stabilizer. The objective of this research was to determine the effect of propolis extract (2%) in the antioxidant status of bovine and porcine patties during chilled storage.

2. Materials and methods

2.1. Preparation of propolis extract

Propolis samples used in this study were collected from an apiary, which was located in Ures, Sonora, Mexico (29.1476 N, -110.1239 O; 632 m). Raw propolis (20 g) were homogenized with ethanol (200 mL) at room temperature (25 °C) for 3 d for further phenolic compound extraction. Propolis extract (PE) then was filtered through Whatman 4 filter paper, concentrated under reduced pressure in a rotary evaporator (BÜCHI R-200, Flawil, Switzerland), washed with hexane to remove waxes, and stored in the dark at −20 °C until analysis (Velázquez, 2007).

2.2. Antioxidant content and antioxidant activities of propolis extract

2.2.1 Total phenolic content

Total phenolic content (TPC) of PE (12.5, 25, 50, 100, 250 and 500 µg/mL) was determined according to the procedure described by Kaur, Arora, & Singh (2008). 100 µL of PE was oxidized with 250 µL of Folin-Ciocalteu reagent (1 N). The mixture was stirred and incubated for 8 min in the dark. Then, 0.750 µL of a solution of Na_2CO_3 (7%, w/v) were added. The mixture was shaken and incubated for 30 min in the dark.

The absorbance was measured at 765 nm in a spectrophotometer (Model 336001, Spectronic Genesys 5, Thermo Electron Corp.). The results are expressed as mg of gallic acid equivalents per g extract (mg GAE/g).

2.2.2 Reducing power

Reducing power assay (RP) was determined using the procedure reported by Geckil et al. (2005). PE (100 µL) was mixed gently with 300 µL of phosphate buffer (0.2 M, pH 6.6), 300 µL of potassium ferric cyanide (1%, w/v) and incubated in a water bath for 20 min at 50 °C. An aliquot of 300 µL of trichloroacetic acid (TCA 10%, w/v) were added and centrifuged for 5 min at 6000 rpm. The supernatant was mixed with 100 µL of distilled water and 250 µL of ferric chloride (0.1%, w/v) and the absorbance was measured at 700 nm in a spectrophotometer. The absorbance increase indicates an increase in the reducing power.

2.2.3 DPPH radical-scavenging activity

The radical-scavenging activity was determined according to Geckil et al. (2005), with slight modifications. The reaction mixture of PE (500 µL) and 500 µL of DPPH solution (300 µM) was incubated 30 min, and absorbance at 517 nm was read. Asc ac was used as standard (100 µg/mL). DPPH radical-scavenging activity (%) was calculated as (Ac−As)×100/Ac, where Ac is the absorbance of the control and As is the absorbance of the sample.

2.3. HPLC-DAD analysis

The HPLC equipment was a Varian ProStar (Walnut Creek, USA) equipped with a diode array detector (DAD), and the stationary phase was a C18 LiChrospher 5 column (125 x 4.0 mm, 5 mm). The flow rate was 1 ml/min and HPLC conditions were as follows: solvent (A) was water-formic acid (5% v/v) and solvent (B) was methanol. The gradient program profile was as follows: starting with 0% B (0 min), 30% (10-20 min), 40% (20-30 min), 45% (30-50 min), 60% (50 -52 min), 80% (52-65 min), 100% (65-70 min) and 0% (70-71 min). The elution of the compounds was monitored at 280 and 340 nm (Hernández et al., 2007).

2.4. Manufacture of bovine and porcine patties

The patties were prepared has described by Vargas-Sánchez et al., (2004) with modifications. Fresh bovine and porcine minced meat 48 hours *postmortem*, were obtained from the local processor and homogenized with 1.5% salt (NaCl, w/w), and 10% fat in final formulation (w/w). A total of 24 patties (25 g/patty) per treatment were formed and placed on Styrofoam™ tray. The polystyrene trays with patties were wrapped with polyvinyl chloride film (17,400 cm^3 O_2/m^2/24 h at 23 °C). The patties were subjected to refrigerated storage at 2 °C in the dark for 0, 3, 6 and 9 d, and 2 packs were opened for subsequent analysis for each formulation. In each replication (twice), bovine and porcine patties were assessed in 8 treatments: (1) B (negative control, bovine patties without additives); (2) B+PE (bovine patties with 2% (w/w) propolis extract); (3) B+BHT (positive control, bovine patties with 0.2% (w/w) BHT; (4) B+Asc ac (positive control, bovine patties with 0.15% (w/w) ascorbic acid); (5) P (negative control, porcine patties without additives); (6) P+PE (porcine patties with 2% (w/w) propolis extract); (7) P+BHT (positive control, porcine patties with 0.2% (w/w) BHT; (8) B+Asc ac (positive control, porcine patties with 0.15% (w/w) ascorbic acid).

2.5. TPC and antioxidant activity in patties

The TPC, RP and DPPH were evaluated using the method of Huang et al. (2011) with slightly modifications. In brief, meat samples (0.05 g) were homogenized rigorously with 500 µL ethanol. After, the samples were subjected to determine the total concentration of phenolic hydroxyl groups, the ability of the meat extraction to donate hydrogen atoms and the presence of reducing agents, respectively.

2.6. Measurement of pH

Meat samples (3 g) were homogenized with 27 mL of distilled water and the pH was measured with a potentiometer (Model pH211, Hanna Instruments Inc., Woonsocket, R.I., U.S.A.) according to Torrescano et al. (2003).

2.7. Measurement of lipid oxidation (TBARS)

Lipid oxidation (Lox) was measured by TBARS-method described by Pfalzgraf, Frigg, & Steinhart (1995). Meat samples (10 g) were homogenized with 20 mL during 1 min, the slurry was centrifuged at 4,500 rpm for 10 min at 5 °C, and filtered through Whatman 4 filter paper. In total, 2 mL of filtrate were added to 2 mL of TBA (0.02 M) in test tubes. The mixture was homogenized, placed in a water bath for 20 min (97 °C), and subsequently cooled. The absorbance at 531 nm was measured. TBARS values were calculated from a 1,1,3,3-tetramethoxypropane standard curve and expressed as mg malondialdehyde/kg of meat sample.

2.8. Measurement of total protein carbonyls

Protein oxidation (Pox), as measured by the total carbonyl content, was evaluated by derivatization with dinitrophenylhydrazine (DNPH) according to the method described by Oliver et al. (1987), with slight modifications. Meat samples (1 g) were homogenized (1:10, w/v) in 20 mM sodium phosphate buffer containing 0.6 M NaCl (pH 6.5) using a a homogenizer (Ultra-Turrax model T25, IKA® Werke GmbH & Co. KG, Janke & Kunkel-Str. 10, 79219 Staufen, Germany) for 30 seg. Two equal aliquots of 0.2 mL were taken from homogenates and dispensed in 2 mL test tubes. Proteins were precipitated with 1 mL of cold TCA (10%, w/v) and subsequently centrifuged at 5,000 rpm for 5 min (one pellet was treated with 1 mL of 2 M HCl for protein concentration measurement and the other with an equal volume of DNPH in 2 M HCl for carbonyl concentration measurement). Both samples were incubated for 1 h at 25 °C. Afterwards, samples were precipitated with 1 mL of TCA (10%, w/v) and washed twice with 1 mL of ethanol:ethyl acetate (1:1, v/v) to remove excess of DNPH. The pellets were then dissolved in 1.5 mL of 20 mM sodium phosphate buffer containing 6 M guanidine HCl (pH 6.5), stirred and centrifuged at 5,000 rpm for 2 min to remove insoluble fragments. Protein concentration was calculated from absorption at 280 nm using BSA as standard. The amount of carbonyls was expressed as nM of carbonyl per milligram of protein using a molar absorption coefficient of 21 nM^{-1} x cm^{-1} at 370 nm for protein hydrazones.

2.9. Measurement of color and metmyoglobin formation

Color changes in the surface of the meat samples during storage were monitored using a CM 2600d spectrophotometer (Konica Minolta Inc., Japan) according to CIE methodology (1978). Values registered were luminosity (L*), red index (a*), yellow index (b*), Chroma (C*) and hue (h*). Before measuring, the samples were extracted from their packing and exposed for 30 min at 5 °C. In total, 5 measurements were performed on the beef patties surface. The metmyoglobin percentage (MetMb%) was determined spectrophotometrically using a CM 2600d (Konica Minolta Inc., Japan) according to Stewart, Zopser, & Walts (1965). The maximum value of the quotient K/S572 and K/S525 at the beginning of the experiment (day 0) was fixed as 0% MetMb, while 100% MetMb was obtained after oxidizing a sample in a potassium ferricyanide solution (1%, w/v). Each value was the mean of 10 measurements on the surface of the beef patties.

2.10. Statistical analysis

All experiments were done by triplicate and the data were subjected to analysis of variance (ANOVA), for assess the effects between treatments in each day of storage (0, 3, 6 and 9 days) and the preservative effect during all storage time (0-9 days). Differences were considered significant at the $P<0.05$ level. If the main effect was significant, the Tukey-Kramer's multiple comparison test was applied at $\alpha=0.05$ (NCSS, 2007).

3. Results and discussion

3.1. TPC, RP and DPPH assay of propolis extract

Phenolic compounds is one of the major groups found in plants and bee products, have been reported to have antioxidant properties (Viuda-Martos et al., 2008; Tan, & Lim, 2015). Determination of TPC, through the Folin-Ciocalteau method and the $AlCl_3$ coloration, is one of important parameters to estimate the amount of antioxidants in extracts from different sources (Geckil et al., 2005). Extract obtained from propolis (PE) was tested "*in vitro*" for TPC assay, and results were influenced ($P<0.05$) by

concentration (Table 1). Propolis at the highest concentration tested (500 µg/mL) presented a high TPC (472.3±3.50 mg GAE/g). Previous research about antioxidant activity of PE suggested that biological activities of propolis from different geographical regions could be due to presence of diverse phytochemicals including phenolic compounds as flavonoids, phenolic acids and their esters (Hernández et al., 2007; Chaillou & Nazareno, 2009). Phenolic compounds can act in many different ways: (1) chelating metals such as iron (Fe^{3+}) and copper (Cu^{2+}), which can prevent their involvement in Fenton reactions that can generate high concentrations of hydroxyl radicals; (2) breaking chain of reactions triggered by free-radicals; (3) and slowing down or accelerating enzyme activity (Biskup, Golonka, Gamian, & Sroka, 2013).

There are numerous methods used for evaluating the antioxidant such as the RP and DPPH (Table 1), which have been widely used and accepted as a model to test the antioxidant properties of PE (Sawaya, da Silva Cunha,& Marcucci, 2011). In this context, results indicate that PE showed high RP at the highest concentration tested (0.56±0.01 abs), compared with synthetic standards (P<0.05). The DPPH activity of PE and synthetic antioxidants (BHT and Asc ac) was evaluated through their ability to quench the DPPH radical. At the highest concentration tested (500 µg/mL) PE presented a high DPPH activity (>70%). Similar values were obtained BHT and Asc ac at 25 µg/mL (P>0.05). Significant correlations were obtained between TPC, RP and DPPH activity (R^2= 0.997), which agree with others investigations (Geckil et al., 2005).Some research indicates that the mechanism followed by phenolic compound to exert their antiradical activity is through the hydrogen atom transfer (HAT), sequential proton-loss electron-transfer (SPLET) and electron transfer followed by proton transfer (SET-PT), which are determined by the solvent properties, radical characteristics, conditions of the medium of reaction, number and position of hydroxyl groups and first reaction OH-site of phenolic compound (Gregoris, & Stevanato, 2010; Biskup, Golonka, Gamian, & Sroka, 2013; Mendoza-Wilson et al., 2013).

3.2. Phenolic composition

Phenolic compounds in PE were identified and reported in Table 2. The main constituents detected in PE were gallic acid, cinnamic acid, p-coumaric acid, naringenin, quercetin, luteolin, kaempferol, apigenin, pinocembrin, pinobanksin 3-acetate, caffeic acid phenethyl ester (CAPE), chrysin, galangin, acacetin and pinostrobin. The flavonoids and phenolic acids constitute a large family of plant phenolic compounds (Tan, & Lim, 2015). The predominant flavonoids compounds were Pinocembrin (130.7±1.8 mg/g), naringenin (50.2±5.9 mg/g) and galangin (37.0±2.1 mg/g), while cinnamic and p-coumaric acid (<3 mg/g) were the phenolic acid more representatives (P<0.05). Similar phenolic composition profile are described for PE collected in Sonoran, Mexico (Hernández et al., 2007). Moreover, relative high levels of flavonoids such as pinocembrin compound are present in propolis from different geographical origins (Cui-Ping et al., 2014). The presence of phenolic compounds in natural extracts are highly correlated with antioxidant activity and natural extracts incorporation, rich in phenolic compounds, to meat and meat products is an important strategy to development of healthier and novel meat products. In this regard several works utilizing fruits, herbs, spices and vegetable extracts, have shown that addition of these extracts to raw and cooked meat products decreased Lox, improved color stability and total antioxidant capacities (Hygreeva, Pandey, & Radhakrishna; Shah, Bosco, & Mir, 2014).

3.3. Antioxidant activity in beef patties

Commonly, *in vitro* activity of meat extracts has been investigated by measuring the Lox stability (Gopalakrishnan, Decker, & Means, 1999). In this context, in this work the antioxidant activity of raw patties was determined in terms of TPC, RP and DPPH activity during the storage time. The results indicate that TPC, RP and DPPH activity was higher for raw bovine (B+PE) and porcine (P+PE) patties treated with PE (Fig. 1A, B and C), which decreased through the storage time (P<0.05). At day 9, TPC of B+PE and P+PE ranged from 75.2±0.6 and 67.3±0.6 mg GAE/g, respectively. In RP values (at day 9), B+PE and P+PE showed values between 0.07±0.01 and 0.09±0.01 abs, which indicate that patties treated with PE show high reducing agents

content than the others treatments (P<0.05). Additionaly, the DPPH assay was applied to measure the radical-scavenging activity in raw patties, and on the last day of sampling (day 9) treatments with PE showed values of antiradical activity close to 45%. The high TPC, RP and DPPH values in raw patties treated with PE could be due to a good phenolic composition showed by PE.

Meat and meat products are not generally considered as a source of dietetic antioxidant (Sacchetti et al., 2008). However, many studies have demonstrated a significantly total phenol contents in beef meat (M. *Longissimus dorsi*, 17.77±0.08 mg GAE/g) and reindeer meat (M. *Longissimus dorsi*, 26.98±0.81; and M. *Semimembranosus*, 29.03±0.81 mg GAE/g) (Mielnik, Rzeszutek, Triumf, & Egelandsdal, 2011). Also, incorporation of fruit purees rich in phenolic content increase significantly the phenolic content (25% compared with the control) in precooked pork breakfast sausage (Leheska et al., 2006); and in goat meat nuggets incorporate with broccoli powder extract (2%) the TPC was 0.16±0.01 mg GAE/g (Banerjee et al., 2012), indicate that natural extracts incorporation can increase the nutritional value of meat without affecting product acceptability. In agreement with our results antioxidant activity has been evaluated in bovine and porcine meat treated with with Lotus rhizome knot (LRK) and Lotus leaf (LL), which increased the DPPH activity in all samples during storage time (Huang et al., 2011); and it has been observed that hydrophilic extracts of poultry meat (lower in total lipids, <1.0 g/100g) increased the DPPH activity than lipophilic, which indicate that solvent polarity could dramatically affect the antioxidant activity of natural compounds and food extracts (Sacchetti, Di-Mattia, Pittia, & Martino, 2008). According to previous investigations the antioxidant stability of meat can be affected by endogenous enzymatic and non-enzymatic antioxidant (Sánchez-Escalante et al., 2001), muscle type (Gheisari, & Motamedi, 2010; Mielnik, Rzeszutek, Triumf, & Egelandsdal, 2011), animal diet (Morán et al., 2012), lipid composition (Ponnampalam et al., 2012), chloride salt content and animal species (Gheisari et al., 2010), thermal treatment (Ma et al., 2007), concentration of natural extract used in meat applications (Banerjee et al., 2012), phenolic content of natural extracts (Leheska et al., 2006), solvent of extraction (Sacchetti et al., 2008), among others.

3.4. Measurement of pH

The pH values of all treatments were initially (day 0) between 5.7 for bovine patties and 5.5 for porcine patties (Fig. 2A), values remained in the range characteristic of fresh bovine meat (Torrescano et al., 2003). The initial pH values of porcine patties are in agree with results obtained by Huang et al. (2011) for raw porcine meat. This results also indicate that incorporation of PE and positive controls not affected the pH values of raw patties, which is not in agreement with Banerjee et al. (2012), who reported that incorporation of broccoli powder extract (2%, w/w) reduce the initial pH values. In this study, pH values of raw patties decreased for all treatments during storage time with remarkable differences (P>0.05), which is in agreement with Vargas-Sánchez et al. (2014).

3.5. Inhibition of lipid oxidation in raw patties

It has been accepted that Lox occurs via free radical chain reaction that proceeds through three steps of initiation, propagation, and termination. Lipid hydroperoxides have been identified as primary products of autoxidation; decomposition of hydroperoxides, aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids, and epoxy compounds, known as secondary oxidation products (Kamal-Eldin, Makinen, & Lampi, 2003). These compounds, together with free radicals, constitute the bases for measurement oxidative deterioration of food lipids. Aldehydic Lox products, especially malondialdehyde (MDA), can be estimated by the reaction with thiobarbituric acid (TBA), since TBARS assay is habitually used as index of Lox in meat products (Raharjo, & Sofos, 1993). Lox in control patties (B and P) increased rapidly during refrigerated storage, reaching 1.0 mg MDA/kg in both meat type (10% fat, w/w), by day 9 (Fig. 2B). The TBARS production was significantly inhibited (P<0.05) in bovine and porcine patties treated with PE (80.0 and 88.7% inhibition, respectively), reaching 0.12 and 0.21 mg MDA/Kg by day 9, values below the acceptable sensory threshold limit (1.0 mg MDA/Kg) for exhibiting rancid flavour (Ockerman et al., 1982). Extracts from propolis have been added to meat products such as cured pork sausage (fat%, unspecified; 5-20°C; 4 weeks) and fresh oriental sausage 14% fat, w/w; 5-°C; 21 days) to reduce Lox and recommended to PE for

extend the shelf life, due its good source of phenolic compounds (Hank, & Park, 2002; Ali, Kassem, & Atta-Alla, 2010). In addition, phenolic compounds are characterized by having in its structure hydrophobic and hydrophilic groups, which allow the molecule interact with non-lipidic and lipidic components (Tsuchiya, 2010). Flavonls (quercetin and rutin) are more effective that flavanones (hesperetin and naringenin) to inhibit MDA production, due to ability to donate H-atoms. However, flavanone naringenin exhibit the deepest interaction with phospholipidic bilayers (Saija et al., 1995). Moreover, 3-hydroxylation of the C ring, non-modification of the B ring and 5,7-dihydroxylation of the A ring led to the greatest membrane interactivity, followed by 3',4'-dihydroxylation of the B ring (Tsuchia, 2010). Our study revealed that Flavanone pinocembrin qualitative and quantitatively is the major component of PE; and its structure having a 5,7-dihidroxylation (Gregoris, & Stevanato, 2010), whereby this compound could be capable to be incorporate easily into cell membranes which increase their structure-dependent membrane interaction and antioxidant potential. The antioxidant effects of phenolic compounds in bio-membranes and meat systems are derived from their ability to scavenge free radicals (via H-atom donation) and through interaction and membrane penetration mechanisms (i.e. modify membrane fluidity), among others (Saija et al., 1995; Tsuchiya, 2010; Rodríguez-Carpena et al., 2011).

3.6. Inhibition of protein oxidation in raw patties

Other components of meat and meat products such as proteins can also be affected by oxidative reactions and consequently cause deterioration of meat quality. The attack of reactive oxygen species (ROS) on muscle proteins result in the loss of sulphydryl groups and the generation of carbonyl compounds. Protein carbonyls can be generated by (1) direct oxidation of amino acid (lysine, arginine and proline) side chains, (2) fragmentation of peptide backbone, (3) reactions with reducing sugars, and (4) binding non-protein carbonyl compounds (Xiong, 2000; Lund et al., 2011). Pox in control patties (B and P) increased gradually during refrigerated storage, reaching >2.0 nM carbonyl/mg in both meat type by day 9. Patties with PE showed a slight significantly decrease in the carbonyl content (<1.3 nM carbonyl/mg) throughout

the storage period ($P<0.05$), regardless meat type (47.3 and 30.6% inhibition, bovine and porcine respectively). Previous studies against Lox have shown that effectiveness of different sources rich in phenolic compounds, is higher than Pox, which may be due to the faster occurrence of lipid oxidation than protein oxidation and interaction between flavonoids and proteins (Arts et al., 2002; Estévez et al., 2008; Rodríguez-Carpena et al., 2011; Gallo, Ferracane, & Naviglio, 2012; Jia et al., 2012; Botsoglou et al., 2014). However, a positive correlation was found between Pox and Lox formation in the present study ($r^2= 0.829$), which indicate that Pox and Lox are timely couple in meat samples (Lund, Heinonen, Baron, & Estevez, 2011).

Some of the phenolic compounds identified in PE such as gallic acid and some flavonols have been described as inhibitors of the formation of carbonyls compounds from myofibrillar proteins (Estévez, & Heinonen, 2010). Also, ferulic acid, malvidin and rutin are characterized for reduced *in vitro* the losses of tryptophan and lysine and consequently Pox, while the chatechins, gallic acid and quercetin had little or no effect on Pox (Heinonen et al., 2010). Grape juice supplementation (rich source of the flavonoids catechin, epicatechin, quercetin and anthocyanidins) reduce the levels of Pox (Byrne, Devaraj, Grundy, & Jialal, 2002). It is noteworthy the lack of correspondence between the antioxidant activity of the extracts in the *in vitro* assays and the activity of such extracts against Pox in patties, which indicate that different system model of analysis may differ considerably. Therefore, more investigations about the effect in vitro and in meat system of each phenolic compounds identified in natural extracts against Pox are required. In general, the addition of PE reduced the Pox, which can be associated to phenolic compound-protein interaction mechanisms mainly related to the hydrophobicity of the aromatic nuclei of polyphenols and the availability of multiple phenolic hydroxyls allowing H-bonding (Hagerman, & Butler, 1981).

3.7. Color parameters and metmyoglobin formation

In meat and meat products, color influences the acceptability and plays a great role in the purchase decision (Girolami, Napolitano, Faraone, & Braghieri, 2013). The changes of L*, a*, b*, C* and h* values of raw patties with and without antioxidant

during chilled storage are shown in Table 3. There was a steady decrease in lightness (L*) and red color (a*) in the controls of bovine a porcine patties, due to prolonged storage of raw patties under oxygen. At day 9, B+PE and P+PE showed a slight significantly decrease in L* (47.20±0.68 and 44.40±0.64, respectively) and a* values (15.0±0.40 and 15.70±0.68, respectively) throughout the storage period ($P<0.05$), indicating a lower loss of color red or pink characteristic of bovine and porcine meat. In order to know the changes in color of fat from the surface patties, yellow index or b* value was evaluated. This value decreased during storage time, and the highest values were found in B+PE and P+PE (17.4±0.50 and 19.10±0.44, respectively) by day 9 ($P<0.05$). The parameter chrome or C* value, has been described as a good indicator that characterizes the color change, because it decreases as the brown color appears, due the MetMb formation, which is favored by lower pH values (Franco *et al*., 2008; Girolami, Napolitano, Faraone, & Braghieri, 2013). At day 9, B+PE and P+PE showed a slight significantly decrease values (23.70±0.50 and 24.4±0.55, respectively) indicating less discoloration of patties respect other treatments. The hue angle or h* value is related to the color and the state of pigments in the muscle, which it is increase as C* value decrease (Franco *et al*., 2008). At day 9, lowest h* values ($P<0.05$) were obtained in treatments B+PE and P+PE (47.70 ± 0.70, 48.40 ± 0.63, respectively), indicating that changes color were lower than other treatments. The discoloration observed in the surface of raw patties can may due to metmyoglobin formation (MetMb%), which In fresh meat Mb is commonly found in three forms (oxymyoglobin, oxy(Fe^{2+})Mb; deoxymioglobin, deoxy(Fe^{2+})Mb; and metmyoglobin, met(Fe^{3+})Mb), is associated to Lox products (Bekhit & Faustmant, 2005; Faustman, Sun, Mancini, & Suman, 2010). There was a steady increase in MetMb (%) in the controls (B and P) during storage ($P<0.05$), reaching unacceptable levels (>40% MetMb) by day 9. B+PE and P+PE showed the lowest values of MetMb (%) during all storage time.

Similary, Jia et al. (2012), found that black currant (*Ribes nigrum* L.) extracts, rich in anthocyannins, enhanced the color of pork patties in a dose-dependent manner. Other phenolic-rich extract such as rosemary, brown seaweed, ulam raja leaves and vine tea, among others, have also reported to inhibit color changes and MetMb

formation of meat samples during chilled storage (Sánchez-Escalante et al., 2001; Bekhit et al., 2003; Moroney, O'Grady, O'Doherty, & Kerry, 2013; Reihani, Tan, Huda, & Easa, 2014; Ye et al., 2015). These results confirm the efficacy of propolis extracts against Lox and Pox, as well as color changes during chill storage.

4. Conclusions

In this study, the current findings demonstrated that the application of PE as an antioxidant in bovine and porcine raw patties stored at 2 °C without illumination can effectively be used to reduce lipid and protein oxidation, as well as color changes and metmyoglobin formation that occur during storage of meat. This work has demonstrated a great potential of PE as preservative for fresh meat products during chilling storage.

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References

Ali, H. F., Kassem, G. M., & Atta-Alla, O. A. (2010). Propolis as natural decontaminant and antioxidant in fresh oriental sausage. *Veterinaria Italiana*, *46*, 167-172.

Arts, M. J., Haenen, G. R., Wilms, L. C., Beetstra, S. A., Heijnen, C. G., Voss, H. P., & Bast, A. (2002). Interactions between flavonoids and proteins: effect on the total antioxidant capacity. *Journal of Agricultural and Food Chemistry*, *50*, 1184-1187.

Banerjee, R., Verma, A. K., Das, A., Rajkumar, V., Shewalkar, A., & Narkhede, H. (2012). Antioxidant effects of broccoli powder extract in goat meat nuggets. *Meat science*, *91*, 179-184.

Bekhit, A. E. D., Geesink, G. H., Ilian, M. A., Morton, J. D., & Bickerstaffe, R. (2003). The effects of natural antioxidants on oxidative processes and metmyoglobin reducing activity in beef patties. *Food Chemistry*, *81*, 175-187.

Bekhit, A. E. D., & Faustmant, C. (2005). Metmyoglobin reducing activity. *Meat Science, 71,* 407-439.

Byrne, D. J., Devaraj, S., Grundy, S. M., & Jialal, I. (2002). Comparison of the antioxidant effects of Concord grape juice flavonoids α-tocopherol on markers of oxidative stress in healthy adults. *The American journal of clinical nutrition*, *76*, 1367-1374.

Biskup, I., Golonka, I., Gamian, A., & Sroka, Z. (2013). Antioxidant activity of selected phenols estimated by ABTS and FRAP methods. *Postepy Hig Med Dosw (Online)*, *67*, 958-63.

Botsoglou, E., Govaris, A., Ambrosiadis, I., Fletouris, D., & Botsoglou, N. (2014). Effect of olive leaf (*Olea europea* L.) extracts on protein and lipid oxidation of long-term frozen *n*-3 fatty acids-enriched pork patties. *Meat Science*, *98*, 150-157.

Brookman, P. (1991). Antioxidants and consumer acceptance. *Food Technology*, 24-28.

CIE. 1978. Recommendations of uniform color spaces-color difference equations psychometric color terms. Supplement No. 2 to CIE Publication No. 15 (E-1.3.1) 1971/(TC-1.3)., Paris: Commision International de l´Eclairage.

Cabrera, M. C., & Saadoun, A. (2014). An overview of the nutritional value of beef and lamb meat from South America. *Meat science*, *98*, 435-444.

Chaillou, L. L., & Nazareno, M. A. (2009). Bioactivity of propolis form Santiago del Estero, Argentina, related to their chemical composition. *LWT–Food Science and Technology, 42,* 1422-1427.

Cui-ping, Z., Shuai, H., Wen-ting, W., Shun, P., Xiao-ge, S., Ya-jing, L., & Fu-liang, H. (2014). Development of High-Performance Liquid Chromatographic for Quality and Authenticity Control of Chinese Propolis. *Journal of Food Science*, 79, C1315-C1322.

Estévez, M., Kylli, P., Puolanne, E., Kivikari, R., & Heinonen, M. (2008). Oxidation of skeletal muscle myofibrillar proteins in oil-in-water emulsions: Interaction with lipids and effect of selected phenolic compounds. *Journal of Agricultural and Food Chemistry*, *56*, 10933–10940.

Estévez, M., & Heinonen, M. (2010). Effect of phenolic compounds on the formation of α-amoniadipic and γ-glutamic semialdehydes from myofibrillar proteins oxidized by cooper, iron, and myoglobin. *Journal of Agricultural and Food Chemistry*, 58, 4448–4455.

Farré, R., Frasquet, I., & Sánchez, A. (2004). El própolis y la salud. *Ars Pharmaceutica*, *45*, 23-43.

Faustman, C., Sun, Q., Mancini, R., & Suman, S. P. (2010). Review: Myoglobin and lipid oxidation interactions: Mechanistic bases and control. *Meat Science*, *86*, 86-94.

Franco, J., Feed, O., Bianchi, G., Garibotto, G., Ballesteros, F., Nan F., Percovich, M., Piriz, M., & Bentancur, O. (2008). Parámetros de calidad de carne en cinco músculos de novillos Holando durante la maduración *post-mortem*. II. Evolución del color durante su almacenamiento. *Agrociencia*, *10*, 69-73.

Gallo, M., Ferracane, R., & Naviglio, D. (2012). Antioxidant addition to prevent lipid and protein oxidation in chicken meat mixed with supercritical extracts of *Echinacea angustifolia*. *The Journal of Supercritical Fluids*, *72*, 198-204.

Geckil, H., Ates, B., Durmaz, G., Erdogan, S., & Yilmaz I. (2005). Antioxidant, free radical scavenging and metal chelating characteristics of propolis. *American Journal of Biochemistry and Biotechnology*, *1*, 27-31.

Gheisari, H. R., & Motamedi, H. (2010). Chloride salt type/ionic strength and refrigeration effects on antioxidant enzymes and lipid oxidation in cattle, camel and chicken meat. *Meat science*, *86*, 377-383.

Girolami, A., Napolitano, F., Faraone, D., & Braghieri, A. (2013). Measurement of meat color using a computer vision system. *Meat science*, *93*, 111-118.

Gopalakrishnan, J., Decker, E.A., & Means, W.J. (1999). Antioxidant activity of mechanically separated pork extracts. *Meat Science*, *52*, 101–110.

Gregoris, E., & Stevanato, R. (2010). Correlations between polyphenolic composition and antioxidant activity of Venetian propolis. *Food and Chemical Toxicology*, *48*, 76-82.

Hagerman, A. E., & Butler, L. G. (1981). The specificity of proanthocyanidin-protein interactions. *Journal of Biological Chemistry*, *256*, 4494-4497.

Han, S. K, & Park, H. K. (2002). Accumulation of thiobarbituric acid-reactive substances in cured pork sausages treated with propolis extracts. *Journal of the Science of Food and Agriculture*, *82*, 1487-1489.

Heinonen, M., Rein, D., Satué-Gracia, M. T., Huang, S. W., German, J. B., & Frankel, E. N. (1998). Effect of protein on the antioxidant activity of phenolic compounds in a lecithin-liposome oxidation system. *Journal of Agricultural and Food Chemistry*, *46*, 917-922.

Hernández, J., Goycoolea, F.M., Quintero, J., Acosta, A., Castañeda, M., & Domínguez, Z. (2007). Sonoran propolis: Chemical composition and antiproliferative activity on cancer cell lines. *Planta Medica*, *73*, 1469-1474.

Huang, B., He, J., Ban, X., Zeng, H., Yao, X., & Wang, Y. (2011). Antioxidant activity of bovine and porcine meat treated with extracts from edible lotus (*Nelumbo nucifera*) rhizome knot and leaf. *Meat Science*, *87*, 46-53.

Hygreeva, D., Pandey, M. C., & Radhakrishna, K. (2014). Potential applications of plant based derivatives as fat replacers, antioxidants and antimicrobials in fresh and processed meat products. *Meat science*, *98*, 47-57.

Jia, N., Kong, B., Liu, Q., Diao, X., & Xia, X. (2012). Antioxidant activity of black currant (Ribes nigrum L.) extract and its inhibitory effect on lipid and protein oxidation of pork patties during chilled storage. *Meat Science*, *91*, 533–539.

Kamal-Eldin, A., Makinen, M., & Lampi, A. M. (2003). Lipid Oxidation Pathways (pp. 1-36). A. Kamal-Eldin, ed., AOCS Press, Champaign, Illinois.

Kaur, R., Arora, S., & Singh, B. (2008). Antioxidant activity of the phenol rich fractions of leaves of Chukrasia tabularis A. *Juss Bioresource Technology*, *99*, 7692-7698.

Kauffman, R. G. (2001). Meat composition. In Y. H. Hui, W. K. Nip, R. W. Rogers, & O. A. Young (Eds.), Meat Science and Applications (pp. 1–19). New York: Marcel Dekker, Inc.

Leheska, J. M., Boyce, J., Brooks, J. C., Hoover, L. C., Thompson, L. D., & Miller, M. F. (2006). Sensory attributes and phenolic content of precooked pork breakfast sausage with fruit purees. *Journal of food science*, *71*, S249-S252.

Löliger, J., & Wille, H. J. (1993). Natural Antioxidants. *Oils & Fats International*, *9*(2), 18-22.

Lund, M. N., Heinonen, M., Baron, C. P., & Estevez, M. (2011). Protein oxidation in muscle foods: A review. *Molecular Nutrition & Food Research*, *55*, 83-95.

Ma, H. J., Ledward, D. A., Zamri, A. I., Frazier, R. A., & Zhou, G. H. (2007). Effects of high pressure/thermal treatment on lipid oxidation in beef and chicken muscle. *Food Chemistry*, *104*, 1575-1579.

Mielnik, M. B., Rzeszutek, A., Triumf, E. C., & Egelandsdal, B. (2011). Antioxidant and other quality properties of reindeer muscle from two different Norwegian regions. *Meat science*, *89*, 526-532.

Morán, L., Andrés, S., Bodas, R., Prieto, N., & Giráldez, F. J. (2012). Meat texture and antioxidant status are improved when carnosic acid is included in the diet of fattening lambs. *Meat science*, *91*, 430-434.

Moroney, N. C., O'Grady, M. N., O'Doherty, J. V., & Kerry, J. P. (2013). Effect of a brown seaweed (*Laminaria digitata*) extract containing laminarin and fucoidan on the quality and shelf-life of fresh and cooked minced pork patties. *Meat science*, *94*, 304-311.

NCSS (2007). Statistical system for windows. Kaysville, Utah, USA: Number Cruncher Statistical Systems.

Ockerman, H. W. (1985). Quality control of post-mortem muscle and tissue. *Department of Animal Science*, Ohio State University. Columbus, OH USA. Vol. 1. p91.

Oliver, C. N., Ahn, B., Moerman, E. J., Goldstein, S., & Stadtman, E. R. (1987). Age-related changes in oxidized proteins. *The Journal of Biological Chemistry*, *62*, 5488-5491.

Pfalzgraf, A., Frigg, M., & Steinhart, H. (1995). A-Tocopherol contents and lipid oxidation in pork muscle and adipose tissue during storage. *Journal of Agricultural and Food Chemistry*, *43*, 1339-1342.

Ponnampalam, E. N., Butler, K. L., McDonagh, M. B., Jacobs, J. L., & Hopkins, D. L. (2012). Relationship between muscle antioxidant status, forms of iron, polyunsaturated fatty acids and functionality (retail colour) of meat in lambs. *Meat science*, *90*, 297-303.

Raharjo, S., & Sofos, J .N. (1993). Methodology for measuring malonaldehyde as a product of lipid peroxidation in muscle tissues: A review. *Meat Science*, *35*, 145-169.

Reihani, S. F. S., Tan, T. C., Huda, N., & Easa, A. M. (2014). Frozen storage stability of beef patties incorporated with extracts from ulam raja leaves (*Cosmos caudatus*). *Food chemistry*, *155*, 17-23.

Rodríguez-Carpena, J. G., Morcuende, D., Andrade, M. J., Kylli, P., & Estévez, M. (2011). Avocado (*Persea americana* Mill.) Phenolics, in vitro antioxidant and antimicrobial activities, and inhibition of lipid and protein oxidation in porcine patties. *Journal of agricultural and food chemistry*, *59*, 5625-5635.

Sacchetti, G., Di Mattia, C., Pittia, P., & Martino, G. (2008). Application of a radical scavenging activity test to measure the total antioxidant activity of poultry meat. *Meat science*, *80*, 1081-1085.

Saija, A., Scalese, M., Lanza, M., Marzullo, D., Bonina, F., & Castelli, F. (1995). Flavonoids as antioxidant agents: importance of their interaction with biomembranes. *Free Radical Biology and Medicine*, *19*, 481-486.

Sánchez-Escalante, A., Djenane, D., Torrescano, G., Beltran, J.A., & Roncales, P. (2001). The effects of ascorbic acid, taurine, carnosine and rosemary powder on colour and lipid stability of beef patties packaged in modified atmosphere. *Meat Science*, *58*, 421-429.

Sawaya, A. C .H .F., da Silva Cunha, I. B., & Marcucci, M. C. (2011). Analytical methods applied to diverse types of Brazilian propolis. *Chemistry Central Journal*, *5*, 27.

Shah, M. A., Bosco, S. J. D., & Mir, S. A. (2014). Plant extracts as natural antioxidants in meat and meat products. *Meat science*, *98*, 21-33.

Stewart, M., Zopser, M., & Walts, B. (1965). The use of reflectance spectrophotometry for assay of raw meat pigments. *Journal of Food Science*, *30*, 464-469.

Tan, J. B. L., Lim, Y. Y. (2015). Critical analysis of current methods for assessing the in vitro antioxidant and antibacterial activity of plant extracts. *Food Chemistry*, *172*, 814-822.

Toldrá, F., & Reig, M. (2011). Innovations for healthier processed meats. Trends in *Food Science & Technology*, *22*, 517–522.

Torrescano, G., Sánchez-Escalante, A., Jiménez, B., Roncalés, P., & Beltrán, J.A. (2003). Shear values of raw samples of 14 bovine muscles and their relation to muscle collagen characteristics. *Meat Science*, *64*, 85-91.

Tsuchiya, H. (2010). Structure-dependent membrane interaction of flavonoids associated with their bioactivity. *Food chemistry*, *120*, 1089-1096.

Valencia, D., Alday, E., Robles-Zepeda, R., Garibay-Escobar, A., Gálvez-Ruiz J.C., Salas-Reyes, M., Jiménez-Estrada, M., Velázquez-Contreras, E., Hernández, J., and Velázquez, C. (2012). Seasonal effect on chemical composition and biological activities of Sonoran propolis. *Food Chemistry, 131*, 645-651.

Vargas-Sánchez, R. D., Torrescano-Urrutia, G. R., Acedo-Félix, E., Carvajal-Millán, E., González-Córdova, A. F., Vallejo-Galland, B., Torres-Llanez, M. J., & Sánchez-Escalante, A. (2014). Antioxidant and antimicrobial activity of commercial propolis extract in beef patties. *Journal of Food Science*, *79*, C1499-C1504.

Velázquez, C., Navarro, M., Acosta, A., Angulo, A., Domínguez, Z., and Robles, R. (2007). Antibacterial and free-radical scavenging activities of Sonoran propolis. *Journal of Applied Microbiology*, *103*, 1747-1756.

Viuda-Martos, M., Ruiz-Navajas, Y., Fernández-López, J., & Pérez-Álvarez, J. A. (2008). Functional properties of honey, propolis, and royal jelly. *Journal of Food Science*, *73*, R117-R124.

Williams, P. (2007). Nutritional composition of red meat. *Nutrition and Dietetics*, *64*, S113-S119.

Wood, J. D., Enser, M., Fisher, A. V., Nute, G. R., Sheard, P. R., Richardson, R. I., & Hughes, S. I. (2008). Fat deposition, fatty acid composition and meat quality: A review. *Meat Science*, *78*, 343–358.

Xiong, Y. L. (2000). Protein oxidation and implications for muscle foods quality. In: Decker EA, Faustman C, and Lopez-Bote CJ, editors. Antioxidants in muscle foods (pp. 85–111). New York: Wiley.

Ye, L., Wang, H., Duncan, S. E., Eigel, W. N., & O'Keefe, S. F. (2015). Antioxidant activities of vine tea (*Ampelopsis grossedentata*) extract and its major component

dihydromyricetin in soybean oil and cooked ground beef. *Food Chemistry*, *172*, 416-422.

Table 1

In vitro antioxidant activity of ethanolic extract from propolis.

PE [µg/mL]	TPC (mg GAE/g)	RP (Abs)	DPPH (%)
500	472.3 ± 3.50*	0.56 ± 0.03*	69.1 ± 0.03*
250	288.1 ± 0.53*	0.34 ± 0.01*	45.7 ± 0.11*
100	198.5 ± 0.98*	0.20 ± 0.01*	33.0 ± 0.01*
50	152.5 ± 1.22*	0.14 ± 0.01*	31.0 ± 0.05*
25	126.6 ± 0.24*	0.10 ± 0.01*	30.0 ± 0.01*
12.5	122.9 ± 0.53*	0.07 ± 0.01*	28.7 ± 0.01*
BHT	ND	0.56 ± 0.01*	70.8 ± 0.10*
Asc ac	ND	0.42 ± 0.01*	73.0 ± 0.10*

ND: not determined. Different concentrations of PE (12.5 to 500 µg/mL) were used in the different assays. BHT (50 µg/mL) and Asc ac (25 µg/mL) were used as antioxidant standards. Data represent means ± respective standard deviations. Significant differences (P<0.05) from control are marked with asterisk.

Table 2

Phenolic compounds identified by HPLC-DAD

Compound No.	Compund	Rt	PE (mg/g)[a]
1	Galic acid	1.9	(+)
2	Cinnamic acid	3.4	2.1 ± 0.2
3	p-coumaric acid	7.8	2.9 ± 0.1
4	Ferulic acid	8.7	(-)
5	Naringenin	27.3	50.2 ± 5.9
6	Quercetin	30.8	6.5 ± 0.2
7	Luteolin	36.4	3.7 ± 0.2
8	Kaempferol	37.2	0.9 ± 0.2
9	Apigenin	40.6	4.4 ± 0.2
10	Pinocembrin	44.5	130.7 ±1.8
11	Pinobanksin 3-acetate	45.8	(+)
12	CAPE	49.0	(+)
13	Chrysin	51.4	12.3 ± 1.0
14	Galangin	52.4	37.0 ± 2.1
15	Acacetin	57.0	8.4 ±0.4
16	Pinostrobin	62.9	(+)

[a] Values are expressed as mean ± standard deviation; Rt, retention time (min); (+), compound identified but no quantified; (-), compound not identified.

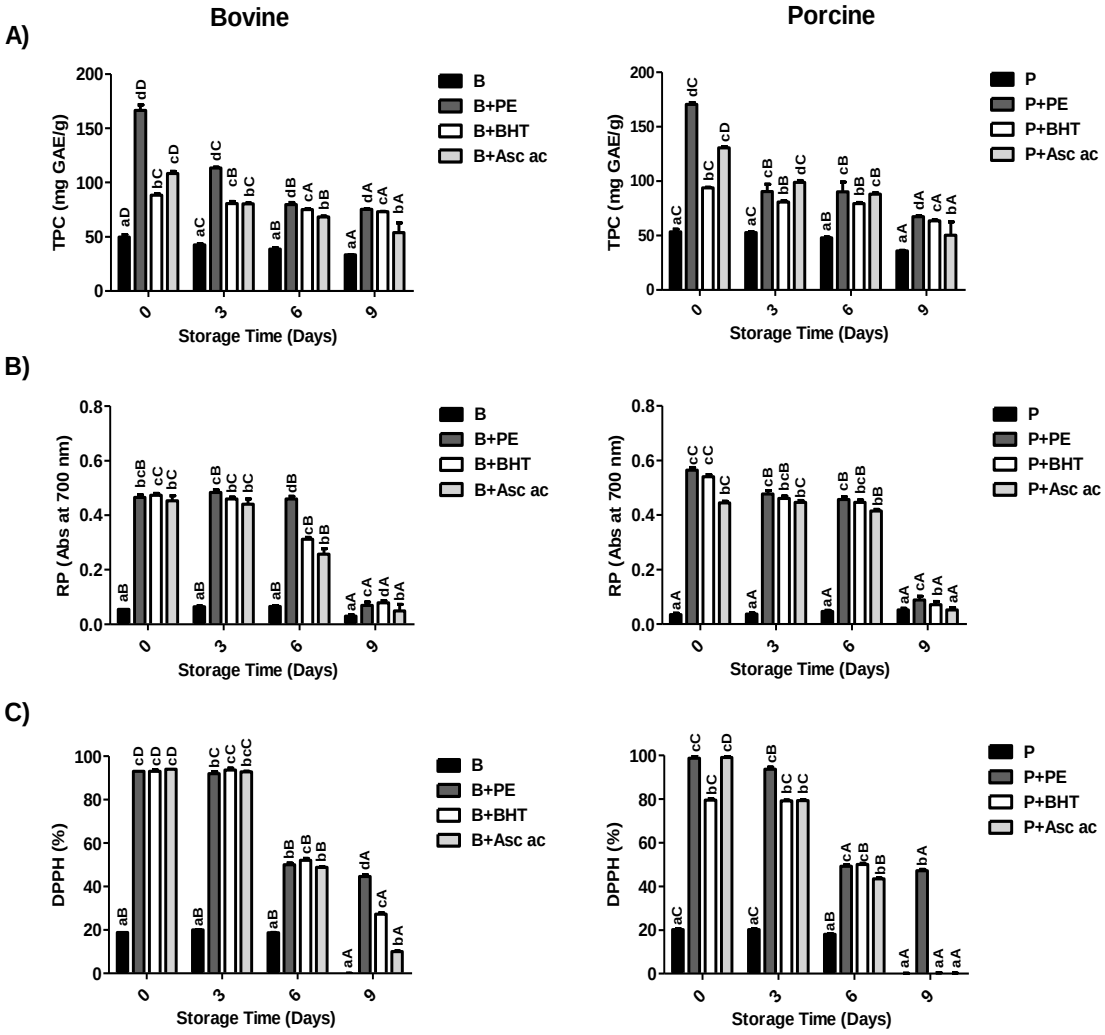

Fig. 1. Antioxidant activity levels in raw patties on storage, as determined by the TPC assay (A), RP assay (B) and DPPH assay (C). Data represent means ± respective standard deviations. Bars with different superscripts (a-d; A-D) within the same sampling day differ significantly (P<0.05).

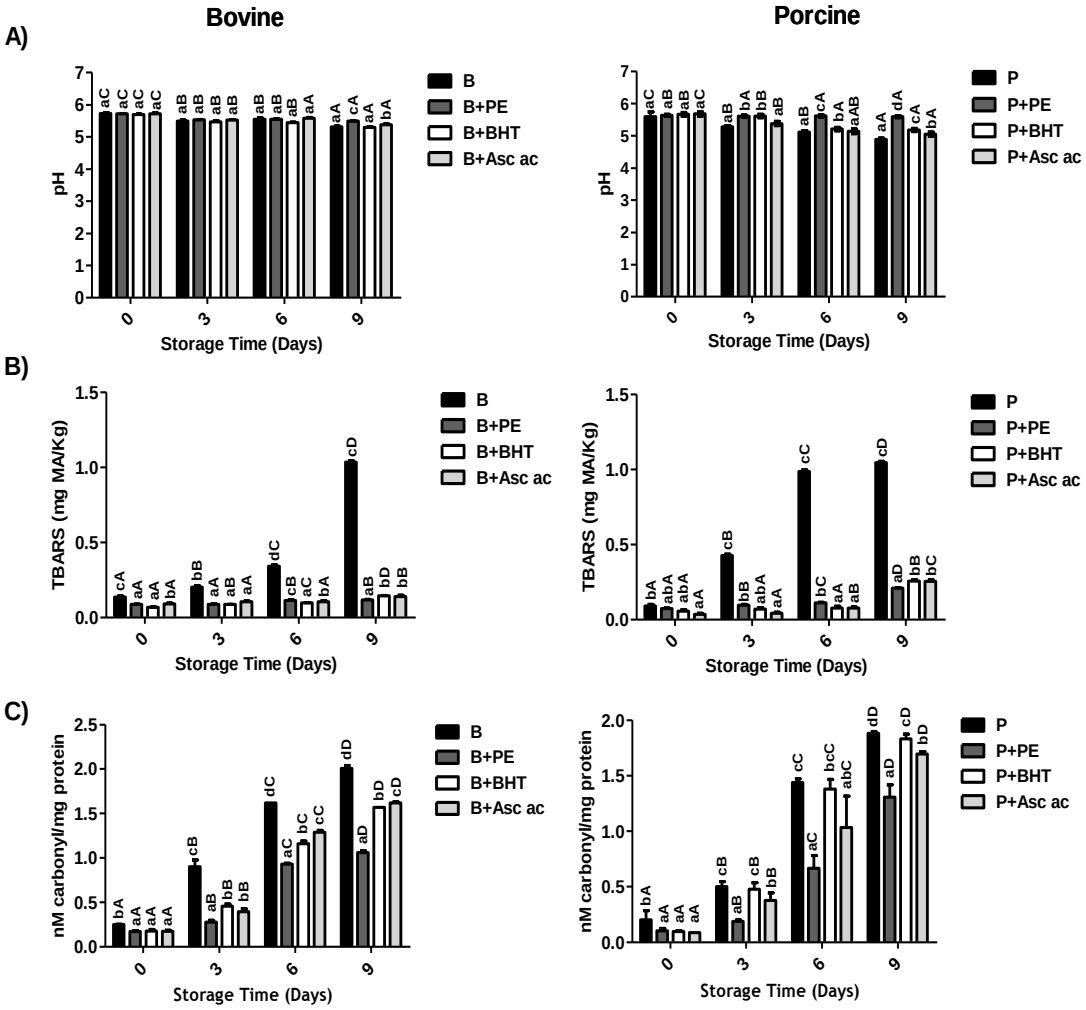

Fig. 2. pH values (A), TBARS values (B) and Protein oxidation levels (C) in raw patties during storage time. Data represent means ± respective standard deviations. Bars with different superscripts (a-d; A-D) within the same sampling day differ significantly (P<0.05).

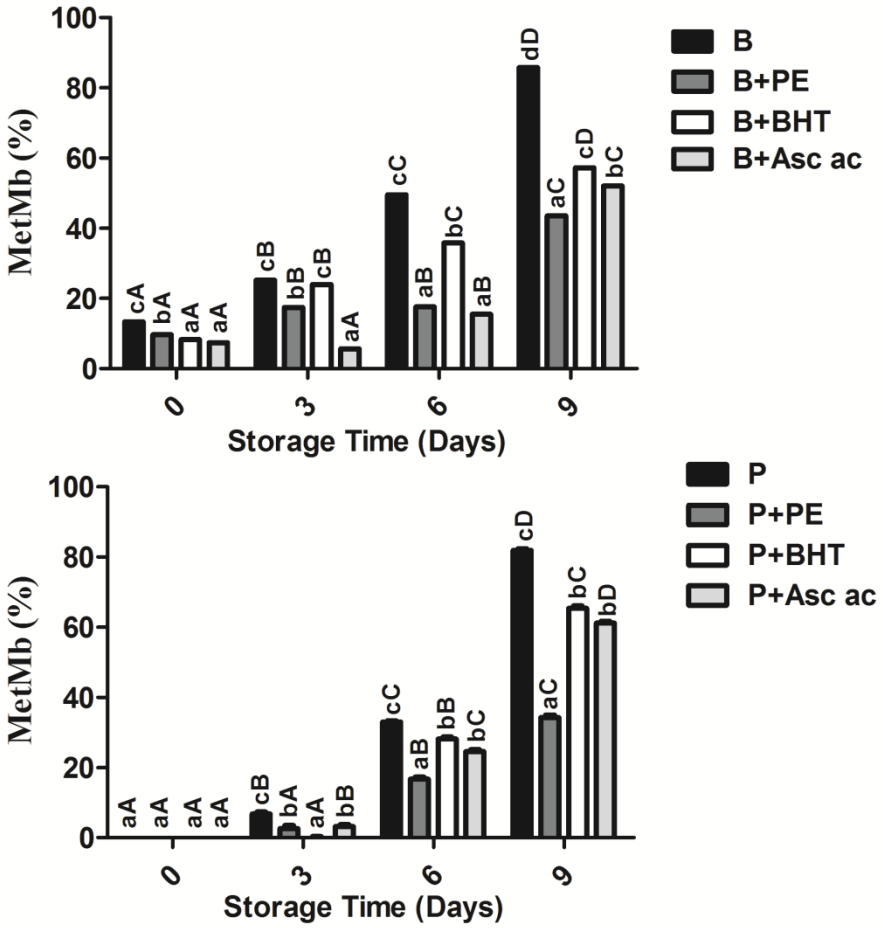

Fig. 3. Metmyoglobin formation in raw patties during storage time. Data represent means ± respective standard deviations. Bars with different superscripts (a-d; A-D) within the same sampling day differ significantly (P<0.05).

Table 2
Instrumental color measured during storage time of bovine and porcine raw patties.

		Bovine				Porcine			
	Day	**B**	**B+PE**	**B+BHT**	**B+Asc ac**	**P**	**P+PE**	**P+BHT**	**P+Asc ac**
L*	**0**	42.0 ± 0.9^{aA}	45.0 ± 0.6^{bA}	44.5 ± 0.6^{bA}	44.7 ± 0.6^{bB}	52.8 ± 1.0^{aA}	56.2 ± 0.8^{bA}	56.4 ± 0.9^{bA}	55.6 ± 0.9^{abA}
	3	41.9 ± 0.8^{aA}	45.9 ± 0.6^{bAB}	45.7 ± 0.6^{bA}	43.6 ± 0.6^{abAB}	52.1 ± 1.0^{aA}	57.2 ± 0.8^{bA}	56.8 ± 1.0^{bA}	56.8 ± 0.7^{bAB}
	6	41.7 ± 1.0^{aA}	47.6 ± 0.7^{cAB}	45.4 ± 0.6^{bcA}	42.8 ± 0.7^{abA}	50.9 ± 0.9^{aA}	59.0 ± 0.8^{bAB}	57.5 ± 1.0^{bA}	59.9 ± 1.2^{bC}
	9	43.0 ± 1.0^{aA}	47.2 ± 0.7^{bB}	45.1 ± 0.6^{abA}	44.4 ± 0.6^{aAB}	49.1 ± 1.0^{aA}	60.6 ± 1.0^{cB}	55.9 ± 0.9^{bA}	59.9 ± 0.9^{cC}
a*	**0**	23.2 ± 0.6^{abC}	22.1 ± 0.4^{aC}	23.0 ± 0.4^{abC}	25.0 ± 0.7^{bC}	20.9 ± 0.5^{aC}	20.4 ± 0.6^{aB}	20.2 ± 0.5^{aC}	19.9 ± 0.6^{aC}
	3	20.9 ± 0.6^{bC}	18.4 ± 0.4^{bB}	20.5 ± 0.4^{bC}	22.2 ± 0.8^{bC}	22.0 ± 0.5^{bC}	17.4 ± 0.6^{aA}	19.8 ± 0.5^{abC}	19.6 ± 0.6^{abC}
	6	11.3 ± 0.6^{aB}	16.0 ± 0.4^{bA}	16.9 ± 0.4^{bB}	19.5 ± 0.7^{cB}	16.6 ± 0.5^{aB}	15.8 ± 0.6^{aA}	17.0 ± 0.5^{aB}	16.1 ± 0.6^{aB}
	9	9.2 ± 0.6^{aA}	15.0 ± 0.4^{bA}	8.1 ± 0.6^{aA}	9.5 ± 0.8^{aA}	11.6 ± 0.6^{aA}	15.7 ± 0.7^{bA}	11.1 ± 0.8^{aA}	10.2 ± 0.6^{aA}
b*	**0**	18.3 ± 0.5^{aC}	20.4 ± 0.5^{aB}	18.5 ± 0.5^{aB}	19.4 ± 0.6^{aC}	17.1 ± 0.6^{aAB}	21.8 ± 0.4^{bB}	18.8 ± 0.4^{aB}	17.6 ± 0.5^{aB}
	3	16.3 ± 0.5^{aB}	17.6 ± 0.4^{aA}	17.3 ± 0.5^{aB}	18.1 ± 0.6^{aC}	18.5 ± 0.6^{abB}	19.7 ± 0.4^{bA}	19.0 ± 0.4^{abB}	17.9 ± 0.5^{aB}
	6	12.6 ± 0.5^{aA}	17.4 ± 0.4^{bA}	15.0 ± 0.5^{bA}	15.3 ± 0.6^{cB}	16.1 ± 0.5^{aA}	19.9 ± 0.4^{bA}	17.4 ± 0.4^{aAB}	17.7 ± 0.5^{aAB}
	9	12.6 ± 0.5^{aA}	17.5 ± 0.5^{cA}	14.3 ± 0.6^{bA}	13.9 ± 0.6^{abA}	16.1 ± 0.5^{aA}	19.1 ± 0.4^{bA}	16.5 ± 0.5^{aA}	15.9 ± 0.5^{aA}
C*	**0**	30.5 ± 0.6^{aC}	29.7 ± 0.4^{aC}	30.4 ± 0.5^{aD}	30.3 ± 0.9^{aC}	26.5 ± 0.7^{aC}	30.1 ± 0.5^{bC}	27.8 ± 0.6^{aC}	27.4 ± 0.5^{aC}
	3	27.0 ± 0.6^{abB}	26.2 ± 0.5^{aB}	26.9 ± 0.5^{abC}	28.9 ± 0.9^{bC}	27.2 ± 0.6^{bC}	27.5 ± 0.6^{abB}	26.6 ± 0.6^{aBC}	25.8 ± 0.5^{aB}
	6	17.0 ± 0.6^{aA}	24.1 ± 0.5^{bA}	23.3 ± 0.5^{bB}	24.8 ± 0.7^{bB}	23.1 ± 0.6^{aB}	26.0 ± 0.6^{bA}	25.0 ± 0.6^{abB}	23.8 ± 0.5^{abB}
	9	15.7 ± 0.6^{aA}	23.7 ± 0.5^{bA}	16.0 ± 0.7^{aA}	17.3 ± 0.8^{aA}	20.2 ± 0.6^{aA}	24.4 ± 0.5^{bA}	20.3 ± 0.7^{aA}	18.7 ± 0.6^{aA}
h*	**0**	38.3 ± 0.5^{aA}	41.9 ± 0.5^{bA}	38.9 ± 0.4^{aA}	37.8 ± 0.5^{aA}	41.7 ± 0.7^{aA}	45.1 ± 0.8^{bA}	42.0 ± 0.6^{aA}	40.7 ± 0.7^{aA}
	3	38.0 ± 0.5^{aA}	43.7 ± 0.5^{cA}	40.2 ± 0.4^{bA}	37.2 ± 0.5^{aA}	42.1 ± 0.5^{aA}	47.0 ± 0.6^{bA}	43.8 ± 0.6^{aA}	43.1 ± 0.7^{aA}
	6	54.8 ± 0.7^{cB}	46.5 ± 0.6^{bB}	40.5 ± 0.5^{aA}	38.2 ± 0.5^{aB}	44.6 ± 0.5^{aB}	49.2 ± 0.5^{bB}	46.1 ± 0.6^{aB}	48.5 ± 0.7^{bB}
	9	60.4 ± 0.7^{bC}	47.7 ± 0.7^{aB}	59.7 ± 0.6^{bB}	59.3 ± 0.7^{bC}	55.9 ± 0.6^{bC}	48.4 ± 0.6^{aB}	55.6 ± 0.9^{bC}	58.1 ± 1.0^{bC}

Data represent means ± respective standard deviations. Bars with different superscripts (a–c; A-D) within the same sampling day differ significantly (P<0.05).

CAPÍTULO III

Pollen profile of Sonoran desert propolis produced in central Sonora, Mexico.

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Pollen profile of Sonoran Desert propolis produced in central Sonora, Mexico

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Summary

Eight propolis samples from the Sonoran Desert were collected at two sites (Pueblo de Álamos, PA; and Rancho Viejo, RV, located at Ures, Sonora, Mexico) during two seasons of the year (winter and summer, 2012 and 2013) and analyzed using palynological methods in order to determine which plants were visited by the bees to collect raw material (resins, wax and pollen) to form the propolis. The pollen analysis indentified a total of 42 pollen types. Six propolis samples were bifloral (summer and winter samples) and two multifloral (winter). *Mimosa distachya var. laxiflora* and *Prosopis velutina* were considered characteristic pollen types with 15 to 45% of total pollen sums..

Keywords: propolis, pollen analysis, botanical origin, Sonora, Mexico

Running head: Propolis pollen contents revealed the plant sources.

1. Introduction

Propolis, a resinous and adhesive natural substance produced by honeybees (*Apis mellifera*), has been used recently in functional foods, and by folk medicine for thousands of years. Previous studies have shown that propolis may have several attributes, including antioxidant, antibacterial, antiviral, antifungal and anticancer properties (Farré et al., 2004; Vargas-Sánchez et al., 2013). The different properties were mainly attributed to phytochemicals such as flavonoids and phenolic acids, which constitute the major component of resins found in propolis samples. The composition of propolis is completed by resins (50%), waxes (30%), volatile essential oils (10%), organic and inorganic chemical compounds (5%), and pollen (5%) (Farré et al., 2004). It is well accepted that the chemical composition and properties of propolis greatly depend on the species of honeybees, season, method of harvesting, and the type of vegetation present in the geographical region (Farré et al., 2004; Peña, 2008). Therefore, it is important to know, through pollen identification, which species have a beekeeping importance, as this can contribute to a more effective distribution of the beehives areas (Santos, 2011; Silva et al., 2013).

The presence of pollen in sediments from Polish propolis was reported by Warakomska and Maciejewicz (1992), who found that the most abundant identified pollen came from plants of the family Brassicaceae, mainly *Brassica*, *Sinapis* and *Raphanus*, which amounted to 9-33%. *Salix* pollen was present in all samples (6-16%), and there were lesser amounts of pollen from *Centaurea* and *Trifolium pratense*. Montenegro et al. (2004) identified various kinds of Chilean propolis based on botanical source, such as propolis from *Escallonia pulverulenta*, *Nothofagus dombeyi*, *Mentha pulegium*, *Eucalyptus*, and *Salix*, among other pollen types. In another investigation in Brazil, Barth and Pinto da Luz (2009), evaluated the presence of pollen grains in seven samples of Brazilian red propolis. The samples were collected from the northeastern coastal region of Brazil. The presence of pollen grains of *Schinus* was considered characteristic in the red propolis sediments. Recently, Matos et al. (2014), determined the pollen spectrum and levels of total phenolic compounds of

propolis samples produced by *Apis mellifera* L. (Apidae) collected from Bahia, Brazil. They found 59 pollen types in propolis samples, being the family Fabaceae the most representative. These works were carried out, in order to characterize the regional vegetation from which the propolis was obtained.

The first investigation of Sonoran Desert propolis (Velázquez et al. 2007), concerned the antibacterial (minimum inhibitory concentration, MIC) and antioxidant (free-radical scavenging, FRS) activities of propolis collected from three different areas. Propolis extracts showed strong antibacterial (mainly gram-positive bacteria at 100 µg/ml) and antioxidant activity (>80% at 100 µg/ml), which were attributed to the presence of flavonoids, and the authors recommended this natural bee product for clinical applications. Sánchez-Escalante et al. (2009) evaluated the antioxidant effect of propolis (produced in the same geographical area) in beef patties as a natural preservative. The results suggest that propolis is a natural strong antioxidant, which can be used in fresh meat products to extend the shelf life. There are numerous reports in the literature indicating that propolis active compounds (mainly organic acids and flavonoids) show antioxidant and antimicrobial properties sequestering free radicals, acting as substrate for radicals such as superoxide and hydroxyl, and metallic ion chelation, while antimicrobial mechanism involves the inhibition of nucleic acids (DNA and RNA) and degradation of cytoplasmic membrane (Vargas-Sánchez et al., 2014).

However, there are no pollen analysis studies of propolis from the Sonoran Desert. The aim of this study was to evaluate the pollen composition of Sonoran Desert propolis obtained from the central Sonoran apicultural area (Ures, Sonora), its relation with the regional vegetation, and the effect of seasonality on the frequency of pollen loads.

2. Material and methods

2.1. Samples and study sites

Propolis samples were collected at two sampling seasons of the year: winter (W; propolis sample made during the period from August to March (2012 and

2013), and summer (S; propolis sample made during the period from March to August (2012 and 2013). The samples were located in the sites known as "Pueblo de Alamos" (PA; 29°8'51.36"N, 110°7'26"W; 636 m) and "Rancho Viejo" (RV, 29°7'19.72"N, 110°16'58.35"W; 476 m), both within the Ures municipality, Sonora, Mexico. This area has an arid to semi-arid climate. Both localities present desert climate characteristics and, in terms of annual rainfall, the local climate is defined as semi-arid (INEGI, 2012). Annual average temperature and rainfall oscillate in the range of 20 to 22 °C and 400 to 500 mm, with a strong seasonality and dominant summer monsoon precipitations (Brito-Castillo et al., 2010; INEGI, 2012; SMN, 2012, 2013). Sampling sites are surrounded by foothills thornscrub, which is dominated by Fabaceae species (Martínez-Yrízar et al., 2010). Eight propolis samples (PAW12, PAW13, PAS12, PAS13, RVW12, RVW13, RVS12, RVS13) were collected from 15 hives per sampling site in four consecutive seasons. Vegetation surveys were conducted in area within 300 m of each collection site, in different seasons, to draw a plant list. The flowering season of the plant list taxa were drawn from published references (Felger et al., 2001; Felger et al., 2013; Hodgson, 2001; Turner et al., 2005)

2.2. Palynological analysis

For pollen extraction, propolis samples (0.5 g) were diluted with 1 ml ethanol and centrifuged at 4000 rpm for 10 min. After cleaning the sample, pollen grains were further dehydrated in glacial acetic acid and prepared for melissopalynological analysis using the acetolysis method (Erdtman, 1960). This consisted in boiling in a water bath (92 °C/10 min) with a mixture of acetic anhydride-sulphuric acid (9:1), then centrifuging, and the supernatant was decanted. Slides were mounted with glycerin and sealed with nail lacquer.

All samples were observed under light microscopy (at x200, x400 and x1000). To determine the frequency classes, 500 pollen grains were counted from each sample. Pollen types were assigned to four percentage classes, as determined by Barth (2004): predominant pollen (>45%); secondary pollen (15 to 45%); important minor pollen (3 to 15%); and minor pollen (<3%). Pollen slides from

the pollen reference collection of Universidad de Sonora-DICTUS (Sonora, Mexico), published pollen atlases, and plant species collected from the sampling areas were used to identify the botanical affinity of the pollen types.

2.3. Statistical analysis

All data were submitted to an analysis of variance (ANOVA) with a post-hoc determination using Tukey's test, and a principal component analysis (PCA) was applied in order to study the correlation between all the variables. The level of significance was set at $P<0.05$ (Software package, SPSS version 21).

3. Results and discussion

3.1. Botanical origin of Sonoran Desert propolis

The vegetation of the sampling zones is dominated by subtropical species (**Table 1**), mainly within the families *Fabaceae* (18.5%), *Cactaceae* (10.2%), *Malvaceae* (8.3%) and *Asteraceae* (6.5%). Fabaceae are an important family of flowering plants, that are known to be the most visited sources for the honey, wax, and propolis production in tropical regions (Almaraz-Abarca et al., 2007; Sebastian-Gernandt, 2010); aside herbs, it includes shrubs and trees, which give the landscape imprint.

Pollen analysis showed a diversity of pollen types in the studied propolis samples. A total of 42 pollen types (**Table 2**), belonging to 22 families, were identified; some of the principal pollen types are illustrated in **Figure 1.** The families with higher pollen frequencies (>15%, <45%) were Fabaceae, Asteraceae, Burseraceae, Chenopodiaceae-Amaranthaceae, and Zygophyllaceae. Highest pollen frequencies were identified in summer samples, corresponding to *Mimosa distachya var. laxiflora* and *Prosopis velutina*: for Pueblo of Alamos, *Mimosa distachya var. laxiflora* (PAS12, 37%; PAS13, 41.6%) and for Rancho Viejo, *Prosopis velutina* (RVS12, 23.1%; RVS13, 29.2%) propolis. A reduction of this pollen types was found in propolis samples produced during the winter, both at Pueblo de Álamos (*Mimosa distachya var. laxiflora*; PAW12, 18.7%; PAW13, 19.4%) and Rancho Viejo (*Prosopis velutina*;

RVW12, 13.3%; RVW13, 16.7%). According to these pollen frequencies, six bi-floral (15 to 45% pollen frequencies: PAW12, PAS12, PAS13, RVW13, RVS12 and RVS13) and two multifloral (<15% pollen frequencies: RVW12 and PAW13) propolis samples were recorded in both regions.

The Sonoran Desert covers a large area including 2/3 of Arizona and much of northwest Mexico, and many trees found in the desert are members of the Fabaceae family. The genus *Mimosa*, which includes *Mimosa distachya var. laxiflora* (Catclaw), is a plant of the Leguminosae (Fabaceae) *Mimosoideae* subfamily, and is native from Northern and Southern America (Robinson, 1989). In Mexico it is mainly distributed in Northern Mexico (Baja California, Chihuahua, Sinaloa and Sonora) and Southern Mexico (Colima, Jalisco, Michoacán, Nayarit and Yucatán) (Molina-Freaner et al., 2010; Sebastian-Gernandt, 2010). Many species of mimosas are economically and socially important, being used in reforestation, timber production, as medicinal plants and for their ability to fix nitrogen. Flowers of several *Mimosa* species are also a major source of nectar for honey production (Simon et al., 2011). The genus *Prosopis*, which includes *Prosopis velutina* (Mesquite), is also within the Leguminosae (Fabaceae) family, *Mimosoideae* subfamily is native from Asian, African and American continents. The genus includes 44 species distributed in arid and semiarid regions (Burkart, 1976). In Mexico it is mainly found in the slopes of the Pacific side, from Michoacán to Oaxaca, and in the Gulf of Mexico, from Nuevo León, Tamaulipas, and northern Veracruz to central regions of the country (Gallegos-Infante et al., 2013). The mesquite is an important plant species for its high nitrogen-fixing potential in very dry areas and drought seasons, and it provides shelter and food to many species of animals and humans, which feed on its leaves, fruits nectar and pollen (Almaraz-Abarca et al., 2007).

The pollen content in propolis samples can have different origins and the pollen types with low and high frequencies can be indicative of the plant species the honeybees used to make the propolis (Silva et al., 2013).The results obtained for Sonoran Desert propolis show that the most predominant pollen types belonged to the more abundant plant sources in the region (Asteraceae,

Malvaceae, Cactaceae and Fabaceae families), and the minor pollen types were obtained from minor plant sources. In all propolis samples the more frequent pollen types were *Mimosa distachya var. laxiflora* and *Prosopis velutina*, which is in agreement with Rivero Montes (2000) and Burboa-Zazueta et al. (2004).

In another investigation, Mohamed and Afaf (2004), reported that the botanical origin of propolis samples from Sudan (Alfaki Hashim, Khartoum State; and Al Abbasia, South Kordofan State), came from more than one plant species; Mimosaceae, Malvaceae and Caesalpiniaceae were the most frequent families of pollen (more than 50 grains) in Alfaki Hashim and Mimosaceae was most abundant in Al Abbasia region. Regarding propolis samples from Al Abbasia: *Mimosa* pigra, *Mimosa* sp, and *Acacia* sp were considered as dominant pollen (16-45%), all belonging to the Fabaceae family. However, in other geographical regions different taxa are recorded in propolis: Pulido-Avila et al. (1998) evaluated the pollen content of a propolis sample from Capilla de Milpillas, Telpatitlan de Morelos (Jalisco, Mexico) to know which plants were used by the bees to collect resins. Pollen analysis revealed a total of 30 pollen types, and *Eucalyptus* sp. (35.2%) and *Fraxinus* sp. (19.9%) were the main genera from which the honeybees obtained the resins. Other minor pollen types (<15%) were Compositae (five pollen types), *Quercus* sp, *Dicliptera* sp, *Bursera* sp, *Citrus* sp., *Mimosa* sp., *Ipomoea* sp., among others. In the same way, Montenegro et al. (2000) revealed that the botanical origin of Chilean propolis is *Eucalyptus*. However, the *Eucalyptus* pollen content (<2%) reported in our work for the central Sonora areas is not in agreement with these studies. In Brazilian regions Santos et al. (2003) established the plant origin of propolis samples, indicating that pollen types representative in all samples were *Schinus*, *Vernonia*, *Diclenia*, *Hyptis*, *Myrcia*, *Weinmania*, *Baccharis* and *Eucalyptus*. In contrast, Moreira et al. (2008), indicated the important contribution of different pollen components such as *Pinus* sp. (0-15%), *Castanea sativa* (0-45%), *Populus tremula* (30-50%) and others (some of them with less than 5%) in propolis from Portugal (Bornes and Fundão regions).

The above differences reflect contrasting vegetation types at these regions.

Pollen depends on the plant diversity at a local and regional scale and these studies show the importance of knowing the pollen content and geographical origin of propolis samples.

3.2. Relationship of propolis samples from different origins.

In order to evaluate the differences among propolis samples collected from different zones and seasons, a multivariate analysis (PCA) was carried out (**Figure 2**). The first principal component (PC1) showed 46.7% of variance and the second principal component (PC2) 26.5%; thus an accumulative 73.2% of total variation was explained by the two first principal components. The results showed a slight separation of the samples analyzed. This was expected because, despite having different geographical origins, the flora of both studied regions is similar. However, propolis samples that showed the highest content of *Mimosa* (PAS13) and *Prosopis* (RVS12) were separated geographically, and seasonally from the other samples ($P<0.05$).

This was also observed in **Table 2**, in which summer propolis from PA showed high levels of *Mimosa,* while summer propolis from RV showed higher contents of *Prosopis* pollen ($P<0.05$), which is in agreement with the local vegetation differences (*Mimosa* is more abundant at Pueblo de Álamos foothills thornscrub, and *Prosopis* in the riparian vegetation at Rancho Viejo), but common flowering periods for *Mimosa distachya* var. *laxiflora* and *Prosopis velutina* (spring to late summer). The high correlations found between propolis samples and the pollen contents (floral origin) is in agreement with Barth and da Luz (2009) who considered a strong correlation among the pollen types, as main characteristic components, and propolis samples. The results of the propolis and pollen taxa PCA (Figure 2) suggests that the first axe displays the geographical origin, while the second axe applies to the seasonality.

Statistical correlation analysis carried out between the meteorological parameters (temperature and rainfall) and the most important pollen types (*Mimosa distachya* var. *laxiflora* and *Prosopis velutina*), showed a significant relationship ($P<0.05$) between temperature, rainfall and pollen contents (**Table**

3). These results show that the flowering species react differentially to temperature and water stress (meteorological conditions), which can affect the pollen composition (Rasmussen, 2002) of propolis.

4. Conclusions

Six propolis samples were found to be bi-floral (i.e. pollen types had secondary percentages, 15-45%), and two multifloral (<15%). Twenty two families and 42 pollen type were found in eight propolis samples. The Fabaceae family was dominant (>45%) in both regions and seasons. Pollen from *Mimosa distachya var. laxiflora* and *Prosopis velutina* were the most representative, and this fact can be explained by the high pollen production of these plants mainly during the second harvesting period (summer), and because these plants are frequently visited by bees. The multivariate analysis was very helpful in the geographical and seasonal differentiation of the propolis samples, particularly those containing high pollen frequencies of *Mimosa* and *Prosopis*. Additionally, the results of this study provide information on other plants from which the bees obtain raw material to form the propolis.

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References

Almaraz-Abarca, N; da Graça-Campos, M; Ávila-Reyes, JA; Naranjo-Jiménez, N; Herrera-Corral, J; González-Valdez, L (2007) Antioxidant activity of polyphenolic extract of monofloral honeybee-collected pollen from mesquite (*Prosopis juliflora*, Leguminosae). *Journal of Food Composition and Analysis*, 20(2): 119–124.

Barth, O M (2004) Melissopalynology in Brazil: A review of pollen analysis of honeys, propolis and pollen loads of bees. *Scientia Agricola*, 61: 342–350.

Barth, O M; da Luz, C F P (2009) Palynological analysis of Brazilian red propolis samples. *Journal of Apicultural Research*, 48(3): 181.

Brito-Castillo, L; Crimmins, M A; Díaz, SCC (2010) Clima. Diversidad biológica de Sonora. Universidad Nacional Autónoma de México, México. pp. 73-96.

Burboa-Zazueta, M G; Goycoolea-Valencia, F; González-Aguilar, G; Torres-Chávez, P I; González-León, A; Hernández-Martínez, J (2004) Identificación y caracterización de compuestos fenólicos presentes en la miel de Mezquite (*Prosopis* ssp) y palo fierro (*Olneya tesota*): marcadores químicos del origen botánico. Centro de Investigación en Alimentación y Desarrollo, A.C. Hermosillo Sonora, México. 133 pp.

Burkart, A (1976) A monograph of the genus *Prosopis* (Leguminosae subfam. Mimosoideae). *Journal of the Arnold Arboretum*, 57(3): 219–249.

Erdtman, G (1960) The acetolysis method: A revised description. *Svensk Botanisk Tidskrif*, 54: 561–564.

Farré, R; Frasquet, I; Sánchez, A (2004) El propolis y la salud. *Ars Pharmaceutica*, 45(1): 21–43.

Felger, R S; Johnson, M B; Wilson, M F (2001) The trees of Sonora, Mexico. Oxford University Press. 400 pp.

Felger, R S; Wilder, B T; Romero-Morales, H (2013) Plant life of a Desert Archipelago **:** Flora of the Sonoran Islands in the Gulf of California, Mexico. University of Arizona Press. 748 pp.

Gallegos-Infante, J A; Rocha-Guzmán, N E; González-Laredo, R F; García-Casas, M A (2013) Efecto del procesamiento térmico sobre la capacidad antioxidante de pinole a base de vainas de mezquite (*Prosopis laevigata*). CyTA-*Journal of Food*, 11(2): 162–170.

Hodgson, W C (2001*)* Food plants of the Sonoran Desert. University of Arizona Press. 313 pp.

INEGI (2012) Instituto Nacional de Estadística y Geografía. Available: http://www.oeidrus-sonora.gob.mx/documentos/Geografia/HISTORIA%20Y%20GEO/URES.pdf

Martínez-Yrízar, A; Felger, R S; Búrquez, A (2010) Los ecosistemas terrestres:

un diverso capital natural. Diversidad biológica de Sonora. Universidad Nacional Autónoma de México, México. pp. 129-156.

Matos, V R; Alencar, S M; Santos, F A R (2014) Pollen types and levels of phenolic compounds in propolis produced by *Apis mellifera* L. (Apidae) in an area of the semiarid region of Bahia, Brazil. *Anais da Academia Brasileira de Ciências*, 86(1): 407–418.

Mohamed, S A E S; Afaf, M E S (2004) Botanical origin of the Sudanese propolis in central areas. *University of Kentucky. Journal of Agricultural Science*, 12(1): 9 pp.

Molina-Freaner, FE; Van-Devender, TR (2010) Diversidad biológica de Sonora. Universidad Nacional Autónoma de México, México. 500 pp.

Montenegro, G; Timmermann, B; Peña, R; Mujica, A; Avila, G (2000) Pollen grains and vegetative structures in propolis as indicators of potencial drugs in Chilean plants, *International Journal of Experimental Botany*, 66: 15–23.

Montenegro, G; Mujica, A M., Peña, R C; Gómez, M; Serey, I; Timmermann, B N (2004) Similitude pattern and botanical origin of the Chilean propolis. *Phyton*, 53: 145–153.

Moreira, L; Dias, L G; Pereira, JA ; Estevinho, L (2008) Antioxidant properties, total phenols and pollen analysis of propolis samples from Portugal. *Food and Chemical Toxicology*, 46(11): 3482–3485.

Peña, R C (2008) Propolis standardization: a chemical and biological review. *Ciencia e Investigación Agraria*, 35(1): 11–20.

Rasmussen, A (2002) The effects of climate change on the birch pollen season in Denmark. *Aerobiologia*, 18(3-4): 253–265.

Rivero Montes, L. K. (2000). Análisis físico-químico y caracterización polínica de miel de abeja en la región central de Sonora. BSc. Thesis, Universidad de Sonora, Hermosillo, Mexico, 126 pp.

Robinson, B. L. (1898). Revision of the North American and Mexican species of *Mimosa*. *Contributions from the Gray Herbarium of Harvard University*, 305-331.

Sánchez-Escalante, A; Vargas-Sánchez, R D; Valenzuela, M; De la Rosa, M A; Torrescano, G; Camou, J P (2009) Evaluation of antioxidant activity of propolis produced in northwest of Mexico for fresh beef patties. Presented at 2009 Annual Meeting of the Institute of Food Technologists. Anaheim, CA, USA.

Santos, F A R (2011) Identificação botânica do pólen apícola. *Magistra*, 23: 5–9.

Sebastian-Gernandt, D (2010) Instituto de Biología. *"Mimosa distachya var. distachya - IBUNAM:MEXU:PVsn1263"*. UNAM. Available: http://unibio.unam.mx/collections/specimens/urn/IBUNAM:MEXU:PVsn1263

Silva, C R B D; Putarov, T; Orsi, R D O (2013) Pollen spectrum of propolis samples from São Paulo State, Brazil. *Acta Scientiarum. Animal Sciences*, *35*(3): 297–300.

Simon, M F; Grether, R; de Queiroz, L P; Särkinen, T E; Dutra, V F; Hughes, C E (2011) The evolutionary history of Mimosa (Leguminosae): toward a phylogeny of the sensitive plants. *American Journal of Botany*, 98(7): 1201-1221.

SMN (2012) Servicio Meteorológico Nacional. Reporte del clima en México 2012. 23 pp.

SMN (2013) Servicio Meteorológico Nacional. Reporte del clima en México 2013. 23 pp.

SPSS Statistics (2012) Statistical Package for the Social Sciences. Chicago, IL, USA.

Turner, R M; Bowers, J E; Burgess, T L (2005) Sonoran Desert plants: an ecological atlas. University of Arizona Press. 504 pp.

Vargas-Sánchez, R D; Torrescano-Urrutia, G R; Sánchez-Escalante, A (2013) El propóleos: conservador potencial para la industria alimentaria. *Interciencia*, 38(10): 705–711.

Vargas-Sánchez, R D; Torrescano-Urrutia, G R; Mendoza-Wilson, A M; Vallejo-Galland, B; Acedo-Félix, E; Sánchez-Escalante, J J; Peñalba-Garmendia, M C; Sánchez-Escalante, A (2014) Mechanisms involved in antioxidant and antibacterial activity of propolis. *Biotecnia*, 16(1): 32–37.

Velázquez, C; Navarro, M; Acosta, A; Angulo, A; Domínguez, Z; Robles, R; Robles-Zepeda, R; Lugo, E; Goycoolea, FM; Velázquez, E F; Astiazaran, H; Hernández, J (2007) Antibacterial and free-radical scavenging activities of Sonoran propolis. *Journal of Applied Microbiology*, 103(5): 1747–1756.

Warakomska, Z; Maciejewicz, W (1992) Microscopic analysis of propolis from Polish regions. *Apidologie*, 23(4): 277–283.

Table 1. Dominant plant taxa in the areas surrounding the study sites, and their flowering seasons.

Acanthaceae	*Dicliptera* sp (Ns; PA, RV) *Elytraria imbricata* (S; PA) *Henrya insularis* (Sp; PA)	*Justicia candicans* (Sp, F, W; PA) *Tetramerium nervosum* (F, W, Sp; RV)
Achatocarpaceae	*Phaulotamnus spinescens* (S, F; PA)	
Agavaceae	*Agave angustifolia* (W, Sp; PA)	
Asteraceae	*Ambrosia* (Sp; PA, RV) *Artemisia* (Ns; RV) *Brickelia coulteri* (Sp, S, F; PA, RV)	*Lagascea decipiens* (F, W, Sp; PA) *Malacothrix sonorae* (W, Sp; PA) *Perityle reinana* (Ns; PA)
Boraginaceae	*Cordia sonorae* (Sp; PA) *Nama hispidum* (Sp; PA, RV)	*Phacelia gentryi* (Sp, S, F, W; PA, RV)
Brassicaceae	*Descurainia pinnata* (W, Sp; PA)	
Burseraceae	*Bursera fagaroides* (S; PA) *Bursera laxiflora* (F, W; PA, RV)	*Bursera microphylla* (S; PA, RV)
Cactaceae	*Cylindropuntia fulgida* (S; PA) *Cylindropuntia leptocaulis* (Sp, S; PA, RV) *Cylindropuntia thurberi* (Ns; PA, RV) *Mammillaria standleyi* (S; PA) *Opuntia engelmannii* (Sp, S; PA, RV)	*Opuntia pubescens* (Ns; PA) *Opuntia* sp (Ns; PA) *Pachycereus pecten-aboriginum* (Sp, S, F, W; PA, RV) *Stenocereus alamosensis* (Ns;PA, RV) *Stenocereus thurberi* (S; PA, RV)
Cannabaceae	*Celtis pallida* (Sp, S, F, W; PA, RV) *Celtis reticulata* (Sp, S; PA, RV)	
Chenopodiaceae	*Chenopodium* sp (Ns; PA, RV)	
Convolvulaceae	*Cuscuta* sp (Ns; PA) *Evolvulus alsinoides* (Sp, S, F, W; PA)	*Ipomoea arborescens* (F, W; PA, RV)
Euphorbiaceae	*Jatropha cardiophylla* (S; RV) *Jatropha cordata* (F; PA, RV)	*Tragia* sp (Ns; PA)
Fabaceae	*Acacia* sp (Ns; PA, RV) *Acacia cochliacantha* (Sp, S; PA, RV) *Acacia constricta* (Sp, S; PA) *Acacia farnesiana* (Sp; PA, RV) *Acacia occidentalis* (S; PA, RV) *Caesalpinia pulcherrima* *(F, W; PA, RV)* *Calliandra eriophylla* (Sp, S; PA) *Coursetia glandulosa* (Sp; PA) *Diphysa suberosa* (S; PA) *Haematoxylon brasiletto* (S; PA, RV) *Havardia mexicana* (Sp; PA, RV)	*Lysiloma divaricatum* (Sp; PA) *Lysiloma watsonii* (S; PA) *Macroptilium* sp (Ns; PA) *Mimosa dysocarpa* (S, F; PA) *Mimosa distachya var. laxiflora* (Sp, S, F; PA, RV)*Nissolia schotti* (S; PA) *Olneya tesota* (Sp; PA, RV) *Parkinsonia praecox* (Sp, S; PA, RV) *Prosopis velutina* (Sp, S, F; PA, RV) *Senna pallida* (Ns; RV)

Seasons (Spring, Sp; Summer, S; Fall, F; Winter, W; Ns, not specified).
Study areas (Pueblo of Álamos, PA; Rancho Viejo, RV).

Table 1. Continued.

Fagaceae	*Quercus* sp (Ns; PA, RV)	
Fouquieriaceae	*Fouquieria macdougalii* (Sp, S;PA, RV)	*Fouquieria splendens* (Sp; PA, RV)
Lamiaceae	*Hyptis albida* (Sp, F, W; PA, RV)	
Malpighiaceae	*Callaeum macropterum* (Sp, S, F, W; PA, RV)	
Malvaceae	*Abutilon* sp (Ns; PA, RV) *Ceiba acuminata* (Ns; PA, RV) *Guazuma ulmifolia* (Ns; RV) *Herissantia crispa* (Sp, S, F; PA)	*Hibiscus* (S; PA) *Sida alamosana* (Ns; PA, RV) *Sphaeralcea* sp. (Sp; RV)
Myrtaceae	*Eucalyptus* sp (Ns; PA, RV)	
Nyctaginaceae	Nyctaginaceae sp (Ns; PA, RV)	
Onagraceae	*Oenothera* sp (Ne; PA)	
Papaveraceae	*Eschscholzia californica* (S; PA)	
Phytolaccaceae	*Phaulothamnus spinescens* (S; PA)	
Poaceae	*Erioneuron pulchellum* (S; PA, RV) *Melinis repens* (Ns; PA, RV)	*Setaria* sp (Ns; PA, RV)
Rhamnaceae	*Karwinskia humboldtiana* (S; PA)	
Rubiaceae	*Hintonia latiflora* (Ns; PA) *Mitracarpus hirtus* (Ns; PA)	*Randia obcordata* (S; PA, RV)
Rutaceae	*Zanthoxylum fagara* (S, F; PA)	
Sapindaceae	*Cardiospermum halicacabum* (Ns; PA, RV)	
Sapotaceae	*Sideroxylon occidentale* (S, F, W; PA)	
Solanaceae	*Capsicum annuum var. glabriusculum* (Ns; PA) *Physalis* sp (Ns; PA)	*Lycium berlandieri* (F, W; PA) *Solanum tridynamum* ((Sp, S; PA)
Scrophulariaceae	*Lycium andersonii* (F, W; PA), *Mimulus floribundus* (Sp, S, F; PA), *Stemodia durantifolia* (Sp, S, F, W; PA)	
Sterculiaceae	*Waltheria* sp (Ns; PA)	
Verbenaceae	*Aloysia gratissima* (Ns; PA)	*Vitex mollis* (Ns; PA)
Viscaceae	*Phoradendron californicum* (W; PA)	
Zygophyllaceae	*Guaiacum coulteri* (S; PA, RV)	
Pteridaceae	*Cheilanthes pringlei* (Ns; PA)	

Seasons (Spring, Sp; Summer, S; Fall, F; Winter, W; Ns, not specified).
Study areas (Pueblo of Álamos, PA; Rancho Viejo, RV).

Table 2. Frequencies of pollen types identified in propolis samples from Pueblo de Álamos (PA) and Rancho Viejo (RV) Sonora, Mexico, collected in winter (W) and summer (S), 2012 (12) and 2013 (13).

Pollen types	PAW12	PAW13	PAS12	PAS13	RVW12	RVW13	RVS12	RVS13
Acanthaceae								
Dicliptera	4.0^{bB}	3.2^{aB}	0.7^{bA}	0.0^{aA}	1.1^{bB}	0.3^{aA}	0.4^{bA}	3.9^{aB}
Agavaceae								
Agave angustifolia	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.2^{bB}
Asteraceae								
Asteraceae	7.0^{aB}	10.0^{bB}	3.7^{aA}	8.6^{bA}	10.5^{bB}	9.8^{aB}	6.9^{bA}	6.0^{aA}
Ambrosia	5.3^{aB}	8.7^{bB}	2.5^{bA}	0.2^{aA}	5.5^{bB}	5.4^{aB}	2.8^{aA}	3.5^{bA}
Artemisia	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.2^{bB}	0.0^{aA}
Boraginaceae								
Nama hispidum	0.0^{aA}	1.3^{bB}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.2^{aB}	0.9^{bB}
Phacelia gentryi	0.3^{bB}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	2.0^{bB}	0.0^{aA}
Burseraceae								
Bursera laxiflora	6.0^{bB}	4.9^{aB}	3.5^{bA}	1.4^{aA}	10.0^{bB}	6.3^{aB}	8.5^{bA}	3.5^{aA}
Bursera microphylla	7.7^{bB}	4.5^{aB}	3.5^{bA}	1.6^{aA}	5.3^{bA}	4.5^{aB}	7.1^{bB}	2.2^{aA}
Cactaceae								
Cactaceae	4.0^{bB}	2.3^{aB}	1.0^{bA}	0.0^{aA}	3.3^{bB}	2.1^{aB}	1.2^{aA}	1.7^{bA}
Cylindropuntia thurberi	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.3^{aA}	0.6^{bB}	1.0^{bB}	0.0^{aA}
Stenocereus alamosensis	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.3^{bB}	0.0^{aA}	0.0^{aA}
Cannabaceae								
Celtis reticulata	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.6^{bB}	0.0^{aA}	0.2^{bA}	0.0^{aA}
Chenop-amaranthaceae								
Chenopodium type	3.7^{aB}	8.7^{bB}	2.7^{bA}	0.2^{aA}	11.1^{aB}	15.2^{bB}	2.4^{aA}	3.5^{bA}
Convolvulaceae								
Cuscuta sp	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.2^{bB}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}
Ipomoea arborescens	2.3^{bB}	0.6^{aA}	1.2^{bA}	0.7^{aB}	1.9^{bB}	1.5^{aB}	0.4^{aA}	0.9^{bA}
Fabaceae								
Acacia sp	5.7^{bA}	5.5^{aA}	11.1^{bB}	6.8^{aB}	5.5^{bB}	4.2^{aA}	4.0^{aA}	4.8^{bB}
Caesalpinia pulcherrima	1.3^{bA}	0.3^{aA}	1.5^{aB}	11.6^{bB}	3.3^{bA}	1.5^{aB}	3.4^{bB}	0.9^{aA}
Havardia type	0.7^{bA}	0.3^{aA}	2.7^{aB}	2.8^{bB}	3.6^{bB}	2.1^{aB}	1.8^{bA}	0.0^{aA}
Haematoxylon brasiletto	0.0^{aA}	0.3^{bB}	0.7^{bB}	0.0^{aA}	0.8^{aA}	0.9^{bA}	2.8^{bB}	1.3^{aB}
Mimosa distachya	18.7^{aA}	19.4^{bA}	37.0^{aB}	41.6^{bB}	13.3^{aA}	16.7^{bA}	21.9^{aB}	25.5^{bB}
Olneya tesota	2.0^{bB}	1.0^{aB}	0.5^{bA}	0.0^{aA}	1.9^{bB}	1.2^{aA}	0.0^{aA}	2.6^{bB}
Parkinsonia praecox	1.0^{aB}	1.0^{aB}	0.5^{bA}	0.0^{aA}	0.6^{bB}	0.3^{aA}	0.0^{aA}	0.6^{bB}
Prosopis velutina	15.3^{bA}	13.6^{aA}	19.8^{bB}	16.0^{aB}	15.2^{aA}	17.3^{bA}	23.1^{aB}	29.2^{bB}

Different superscripts (a–b) within the same sample and season differ significantly ($P<0.05$). Different superscripts (A–B) within the same year of collection and different season differ significantly ($P<0.05$).

Table 2. Continued.

Pollen types	PAW12	PAW13	PAS12	PAS13	RVW12	RVW13	RVS12	RVS13
Fagaceae								
Quercus sp	0.0^{aA}	0.0^{aA}	0.2^{aB}	0.4^{bB}	0.8^{aB}	1.5^{bB}	0.0^{aA}	0.0^{aA}
Fouquieriaceae								
Fouquieria	0.0^{aA}	0.6^{bA}	0.2^{aB}	2.8^{bB}	0.0^{aA}	0.0^{aA}	0.2^{aB}	0.4^{bB}
Lamiaceae								
Hyptis albida	0.3^{bA}	0.0^{aA}	1.5^{bB}	0.0^{aA}	0.8^{bB}	0.3^{aB}	0.6^{bA}	0.0^{aA}
Malvaceae								
Abutilon	2.0^{aB}	2.9^{bB}	0.0^{aA}	0.0^{aA}	1.1^{aB}	1.5^{bB}	0.4^{bA}	0.0^{aA}
Ceiba acuminata	0.0^{aA}	0.6^{bB}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}
Herissantia crispa	0.0^{aA}	2.9^{bB}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}
Hibiscus (type)	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.2^{bB}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}
Sida alamosana	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.2^{bB}	0.0^{aA}	0.0^{aA}	0.2^{bB}	0.0^{aA}
Sphaeralcea	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.8^{bB}	0.0^{aA}
Myrtaceae								
Eucalyptus sp	0.0^{aA}	1.6^{bB}	0.7^{aB}	0.9^{bA}	1.4^{bB}	1.2^{aB}	0.4^{aA}	0.7^{bA}
Nyctaginaceae								
Nyctaginaceae sp	0.7^{bB}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.3^{aB}	0.3^{aA}	0.0^{aA}	2.2^{bB}
Poaceae								
Poaceae sp	2.8^{bB}	1.6^{aB}	0.0^{aA}	0.5^{bA}	0.3^{aA}	0.9^{bA}	2.8^{aB}	2.6^{bB}
Rhamnaceae								
Karwinskia	1.7^{bB}	0.6^{aB}	0.5^{bA}	0.0^{aA}	0.0^{a}A	0.0^{aA}	0.0^{aA}	0.0^{aA}
Rubiaceae								
Mitracarpus hirtus	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.2^{bB}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}
Randia obcordata	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.8^{bB}	0.0^{aA}
Randia sp	2.7^{bB}	0.0^{aA}	0.5^{bA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}	0.0^{aA}
Sapindaceae								
Cardiospermum	0.7^{bB}	0.6^{aA}	0^{aA}	1.1^{bB}	0.3^{bA}	0.0^{aA}	0.8^{bB}	0.7^{aB}
Zygophyllaceae								
Guaiacum coulteri	3.7^{bB}	1.3^{aB}	1.5^{bA}	1.1^{aA}	0.8^{aA}	2.7^{bB}	2.4^{bB}	1.3^{aA}
Not identified	0.7	1.3	2.2	1.2	0.3	1.8	0.6	0.6
Total of pollen types (%)	100	100	100	100	100	100	100	100

Different superscripts (a–b) within the same sample and season differ significantly (P<0.05). Different superscripts (A–B) within the same year of collection and different season differ significantly (P<0.05).

Table 3. Correlation between the most representative propolis pollen taxa from central Sonoran Desert and values of average annual temperatures and rainfall.

	Mimosa	***Prosopis***
2012		
Temperature	0.483	0.976
Rainfall	0.809	0.915
2013		
Temperature	0.548	0.832
Rainfall	0.836	0.935

Temperature in °C.
Rainfall in mm.

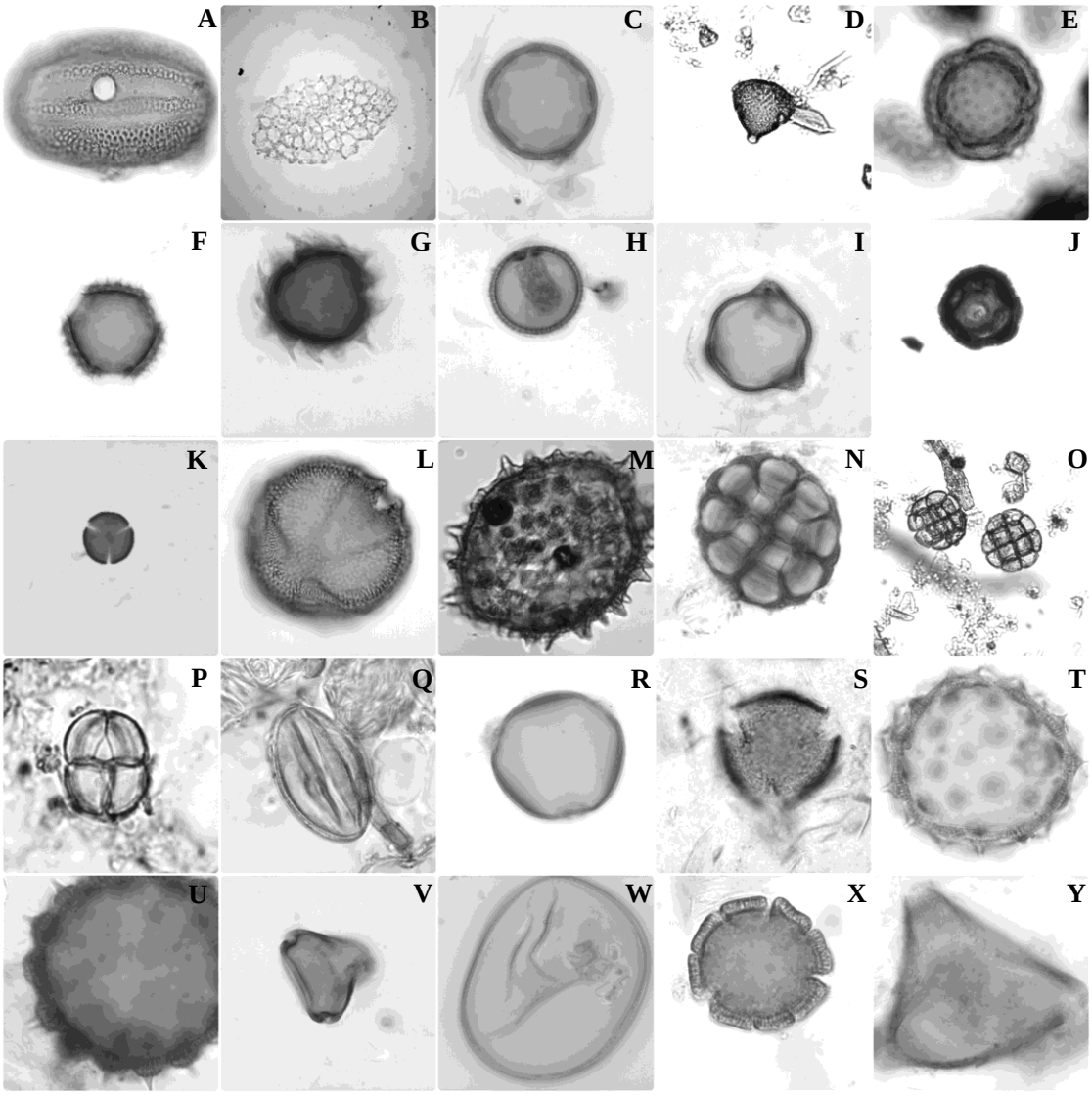

Figure 1. Some pollen types found in the Sonoran Desert propolis. Different scales are indicated for each pollen type. Acanthaceae: *Dicliptera* (**A**, 1000x). Agavaceae: *Agave angustifolia* (**B**, 400x). Chenopodiaceae-Amaranthaceae: *Chenopodium* sp (**C**, 1000x), Malvaceae: *Ceiba acuminata* (**D**, 200x). Asteraceae: *Ambrosia* (**E**, 1000x), Asteraceae (**F**, 1000x), Asteraceae (**G**, 1000x). Burseraceae: *Bursera laxiflora* (**H**, 1000x), *Bursera microphylla* (**I**, 1000x). Cactaceae: *Cylindropuntia thurberi* (**J**, 200x), *Stenocereus alamosensis* (**K**, 200x). Fabaceae: *Caesalpinia pulcherrima* (**L**, 1000x). Convolvulaceae: *Ipomoea arborescens* (**M**, 400x). Fabaceae: *Acacia* sp (**N**, 1000x), *Havardia* type (**O**, 400x), *Mimosa distachya var.* L (**P**, 1000x), *Olneya tesota* (**Q**, 1000x), *Prosopis velutina* (**R**, 1000x). Fagaceae: *Quercus* sp (**S**, 1000x). Malvaceae: *Abutilon* (type, **T**, 1000x), *Herissantia crispa* (**U**, 1000x). Myrtaceae: *Eucalyptus* sp (**V**, 1000x). Poaceae sp (**W**, 1000x). Rubiaceae: *Mitracarpus hirtus* (**X**, 1000x). Sapindaceae: *Cardiospermum halicacabum* (**Y**, 1000x).

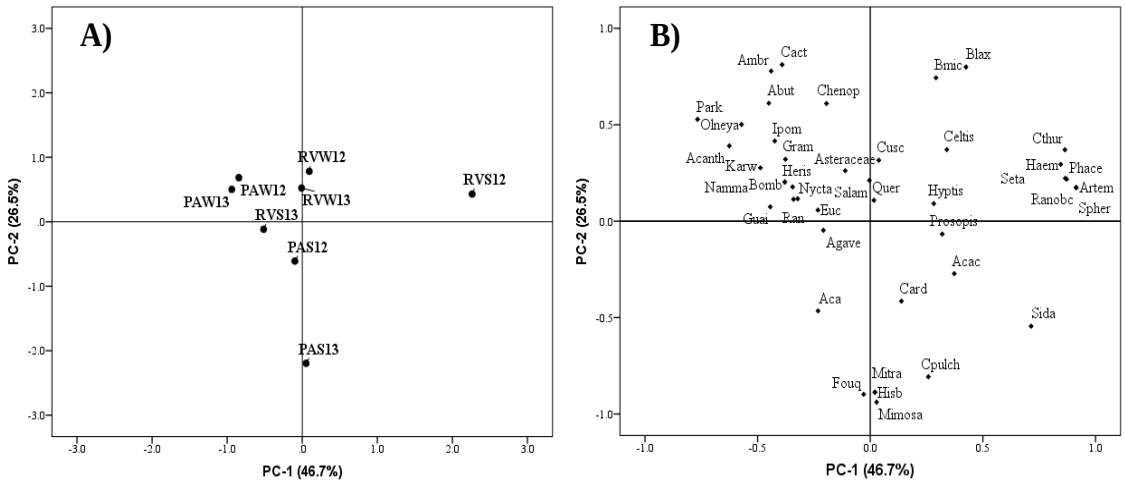

Figure 2. Principal components analysis of propolis samples (A) and pollen taxa (B).

CAPÍTULO IV

Effect of pollen source on organoleptic, physicochemical, chemical composition and biological activities of propolis.

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Effect of pollen source on organoleptic and physicochemical properties, phenolic composition and biological activities of propolis.

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ABSTRACT

Propolis samples were analyzed to determined the relationship between pollen source, organoleptic and physicochemical characteristics, phenolic composition and biological activities of propolis collected at different season and provenances from the Northwest of Mexico. Antioxidant activity was expressed in terms of total phenolic content (TPC), Flavones and flavonols (FF), Flavanones and dihydroflavonols (FD) and Ferric reducing antioxidant power (FRAP). Antibacterial activity was determined against two Gram-positive bacteria (*Staphylococcus aureus* and *Listeria innocua*) and two Gram-negative bacteria (*Echerichia coli* and *Salmonella thyphimurium*). The results indicate that Mesquite and Catclaw pollen sources were predominant in propolis samples. Fifteen phenolic compounds (gallic acid, cinnamic acid, p-coumaric acid, naringenin, quercetin, luteolin, kaempferol, apigenin, pinocembrin, pinobanksin 3-acetate, CAPE, chrysin, galangin, acacetin and pinostrobin) were identified by HPLC-DAD. Propolis rich in Mesquite pollen showed the highest antioxidant and antibacterial properties, which indicate that pollen source marks differences in propolis from different botanical origin.

***Keywords*:** Propolis, Pollen source, propolis quality, phenolic composition, biological activities.

1. Introduction

Propolis (CAS 9009-62-5) is a resinous material that is collected by honeybees (*Apis mellifera*) from exudates of several trees and plants and mixed with pollen and wax by bee enzymes. The word propolis (from the Greek pro "in defense or for" and polis "city") reflects its importance to bees, since they use it to block holes and crack, embalming invaders that bees kills, and protect the colony from diseases (Farré, Frasquet, & Sánchez, 2004; Bankova, 2005). Raw propolis composition is complex and the main constituents of propolis are resins (50-55 %), waxes (25-35 %), essential oils (10 %), pollen (5 %) and other substances (5 %), such as minerals and phenolic compounds (Medana, Carbone, Aigotti, & Appendino, 2008).

Numerous studies have reveled that propolis possessed a broad spectrum of pharmacological activities including antitumoral, anti-inflammatory, antioxidant, antibacterial, antiviral, antifungal, and antiparasite properties among others (Bankova, 2005; Lotfy , 2006; Valença et al., 2013), which are correlated with its phenolic composition, as well as flavonoids (apigenin, chrysin, galangin, hesperetin, kaempferol, luteolin, myricentin, pinocembrin, pinobanksin, pinostrobin, and quercetin) and phenolic acids (caffeic, p-coumaric, and ferulic). This organic compounds are synthesized by plants as secondary products serving in plant defense mechanisms, and characterized by having in their structure at least one phenol group, an aromatic ring attached to at least one hydroxyl functional group, also to be strongly associated with the biological properties of propolis (Martínez-Florez, González-Gallego, Culebras, & Tunon, 2002; Trusheva, Trunkova, & Bankova, 2007; Vargas-Sánchez et al., 2014).

Quality properties of propolis such as organoleptic and physicochemical characteristics, as well as biological activities have been used to classified propolis from different geographical regions ( ), while phenolic composition of several types of propolis has been regarded as a important tool to determined the botanical origin of propolis, resin and pollen content ( ). For example, propolis samples collected from different regions with organoleptic (color, flavor and odor) and physicochemical characteristics (resin, wax and mechanical

impurities) within of permissible limits, increase product quality in terms of total phenolic and flavonoids content and biological activity (Chaillou, Herrera, & Maidana, 2004). Respect botanical origin, phenolic compounds such as izalpinin, 2',4'-dihydroxy-3'-methoxychalcone, 2',4'-dihydroxychalcone, 7-hydroxyflavanone, galangin, pinocembrin, rhamnocitrin, 3-hydroxy-7,8-dimethoxyflavone, 7-hydroxy-8-methoxyflavanone and pinostrobin have been associated with Caesalphineae plants (Agüero et al., 2009) and more recently 2',3',4'-trimethoxychalcone, 2'-hydroxy-3',4'-dimethoxychalcone, 2',4'-dihydroxy-3'-methoxychalcone, pinobanksin 3-acetate and 5,7-dihydroxy-6-methoxy-2,3-dihydroflavonol 3-acetate have been correlated with *Acacia paradoxa*, which is a plant endemic to the region of collection (Tran, Duke, Abu-Mellal, & Duke, 2012).

The pollen composition could also play an important role in biological properties of propolis extracts. For example, it has been documented that propolis associated with *Populus tremula* (*Populus tremula*, 30-50%; *Castanea sativa*, 0-45%; *Pinus* sp., 0-15%; others, 0-35%) increase e total phenolic content and antioxidant activity (Moreira, Dias, & Pereira, 2008) and recently has been reported that bee pollen from Myrtaceae eucalyptus, Asteraceae and Brassicaceae families, rich in phenolic compounds (rutin and myricetin), increase the antioxidant activity. In another study performed by LeBlanc, Davis, Boue, DeLucca, & Deeby (2009) bee pollen from Sonoran desert showed high antioxidant activity, which was correlated to some phenolic compounds. Several types of propolis has been characterized under different geographical, climatic and resin plant source. However, in relation to its pollen source and how this affects their organoleptic and physicochemical attributes, chemical composition and biological activities has not been analyzed.

The present work was conducted to determine the effect of pollen source on the organoleptic and physicochemical characteristics, phenolic composition, and biological properties of propolis, collected in the Norwest of Mexico.

2. Materials and methods

2.1. Standards and reagents

All chemicals used were of analytical grade. Folin–Ciocalteu's reagent, sodium carbonate (Na_2CO_3), gallic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), formic acid (HPLC purity), methanol (HPLC purity), ethanol, hexane, iron(III) chloride 6-hydrate ($FeCl_3$ $3H_2O$), iron(II) sulfate 7-hydrate ($FeSO_4$ $7H_2O$) and acetic acid (CH_3COOH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The following authentic standards of phenolic compounds: acacetin, caffeic acid phenethyl ester (CAPE), cinnamic acid, chrysin and galangin were obtained Sigma Chemical Co. (St. Louis, MO, USA). Hesperetin, naringenin, kaempherol, pinocembrin and 7-methoxy pinocembrin were purchased from INDOFINE (Chemical company, inc., Hillsborough, NJ). 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) was purchased from Fluka Chemie AG (Bushs, Switzerland). Hydrochloric acid (HCl) and methanol were obtained from Merck (Darmstadt, Germany). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman carboxylic acid), potassium persulphate ($K_2S_2O_8$), and gallic acid were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Sample collection and extract preparation

Propolis samples were collected from Apis mellifera beehives in Sonora (Norwest of Mexico; 29.1476 N, -110.1239 O; 632 m) in two main sampling seasons of the year, winter (from December 22 to March 20, 2012 and 2013), and summer (June 22 to September 22, 2012 and 2013). Raw propolis (20 g) were cut into small pieces and extracted with ethanol (200 mL) during 3 days. Then the extracts were filtered through Whatman No. 4 filter paper and concentrated under reduced pressure in a rotary evaporator (BÜCHI R-200, Switzerland). The propolis extract (PE) was washed with n-hexane to remove waxes, lyophilized and stored at –20 °C under darkness until analysis (Hernández et al., 2007).

2.3. Pollen analysis

The pollen identification was performed according to the acetolysis method (Barth, Dutra, & Justo, 1999). The pollen examination (ca. 500 grains per sample) was performed by optical microscope at 40x magnification. Pollen identification was based on the reference collection from the Laboratory of Palynology, Dictus Dept., USON, Mexico., and different pollen morphology obtained from plant collection were used for the recognition of the pollen types.

2.4. Organoleptic and physicochemical characteristics

The organoleptic characterization was carried out as described (NSO, 2003; Lozina, Peichoto, Acosta, & Granero, 2010). The physicochemical analyses was performed according (Lozina, Peichoto, Acosta, & Granero, 2010). In moisture and ash content of propolis samples (5 g) were dried in an oven at 100 °C/8 h and incinerate in a muffle at 550 °C/3 h, respectively. The waxes and mechanical content of dry propolis samples (5 g) were extracted under reflux with petroleum ether. After remove the waxes, the sample was placed in an oven at 100 °C/3 h and cooled until constant weight. In mechanical impurities determination, propolis samples were homogenized with n-hexane and ethanol at 40 °C/3 days. The residue was filtered and dried (100 °C/3 h) until constant weight. The results were determined gravimetrically.

2.5. HPLC-DAD analysis

The HPLC equipment was a Varian ProStar (Walnut Creek, USA) equipped with a diode array detector. Stationary phase was a C18 LiChrospher 5 column (125 x 4.0 mm, 5 mm). The column was eluted using a water-formic acid/methanol gradient at a flow rate of 1 mL/min. The mobile phase consisted of 5% formic acid in water (A) and methanol (B). The gradient program was 30% B (0-15 min), 40% B (15-20 min), 45% B (20-30 min), 60% B (30-50 min), 80% B (50-65 min), 100% B (65-75 min). The elution of each peak was monitored at 280 and 340 nm (Hernández et al., 2007).

2.6. Total phenolic content

Total phenolic content (TPC) in PE was evaluated by using the Folin-Ciocalteu method (Popova et al., 2005) with slight modifications. Briefly, 10 µL of PE (5 mg/mL) were transferred into each well of flat 96-well microplate, diluted with 80 µL of distilled water and 40 µL of Folin–Ciocalteu's reagent (0.25 N) and 60 µL of Na_2CO_3 (7%) solution were added. The reaction mixture was homogenized with 80 µL of distilled water and incubated in the dark (at room temperature, 25 °C) during 1 h. The absorbance was measured at 750 nm in a spectrophotometer (Multiskan FC UV-Vis, Thermo Scientific). The total phenolic content was expressed as mg pinocembrin/galangin equivalent per g of propolis.

2.7. Flavones and flavonols content

The total flavones and flavonols content (FF) of PE was determined by using the aluminum chloride complex formation method (Popova et al., 2004) with slight modifications. Briefly, 10 µL of PE (5 mg/ml) were transferred into each well of flat 96-well microplate, diluted with 130 µL of methanol and 10 µL of $AlCl_3$ (5%) were added. The mixture was incubated for 30 min (at room temperature). The absorbance was read at 412 nm in a spectrophotometer. The results were expressed as mg quercetin equivalent per g of propolis.

2.8. Flavanone and dihydroflavonol content

The flavanone and dihydroflavonol contents (FD) of PE were determined by the method as described Popova et al. (2004) with slight modifications. Briefly, 40 µl of PE (5 mg/Ll) were transferred into each well of flat 96-well microplate, mixed with 80 µL of 2,4-dinitrophenylhydrazine (DNP) solution [50 mg DNP in 100 µL of sulphuric acid 96 % (v/v), diluted to 10 mL with methanol] and heated at 50 °C for 50 min. After cooling, the mixture was diluted with 280 µL of KOH (10%) in methanol (w/v) and 30 µL of the resulting solution were diluted with 250 µL of methanol. The absorbance was measured at 490 nm in a spectrophotometer. The results were expressed as mg of pinocembrin equivalents per g of propolis.

2.9. Ferric reducing antioxidant power

The ferric reducing-antioxidant power (FRAP) was performed as described Benzie & Strain (1999). 5 µL of PE (5 mg/ml) were transferred into each well of flat 96-well microplate, and homogenized with 150 µL of FRAP solution [10:1:1, buffer sodium acetate 300 mM in glacial acetic acid (pH 3.6), 4, 4, 6-tripyridyl-S-triazine (TPZ) 10 mM in 40 nM HCl and $FeCl_3$ 20 mM]. The resulting solution was incubated during 8 min in the dark and absorbance was measured at 595 nm in a spectrophotometer. The reported values are expressed in terms of the amount of the ferrous form of [Fe^{2+}] produced from a standard curve plot of ferrous ascorbate in mg/mL, as the ferric form is reduced. The results were expressed as µmol Fe(II) per g propolis.

2.10. Free-radical scavenging activity

Free-radical scavenging activity (FRS) was carried out according as described Hatano, Kagawa, Yasuhara, & Okuda (1988) with slight modifications. 100 µL of PE (100 µg/mL) were transferred into each well of flat 96-well microplate, and mixed with an equal volume of DPPH solution (300 µmol, [2,2-diphenyl-1-pichrylhydrazyl]). The resulting solution was homogenized and after 30 min of incubation in the dark, absorbance was measured at 520 nm in a spectrophotometer. Vitamin C, TROLOX and Caffeic acid phenethyl ester (CAPE) were used as antioxidant standards (25 µg/mL). The FRS was calculated by the following equation:

$$FRS\ (\%) = \left[\frac{1 - \ Abs\ (sample)}{Abs\ (0)}\right] x\ 100$$

Where Abs (0) is the absorbance of the control at t = 0 min, and Abs (sample) is the absorbance of the antioxidant at t = 60 min.

2.11. Antibacterial activity

In vitro antibacterial studies were performed by the broth microdilution method as described by Jorgensen, Turnidge, & Washington (1999), with slight

modifications. *Staphylococcus aureus* ATCC 29213B, *Listeria innocua*, *Escherichia coli* ATCC 25922 and *Salmonella typhimurium* ATCC 14028 strains were initially reactivated in liquid nutrient broth (BHI agar) for 24-48 h at 37 °C. After bacterial growth an aliquot of 50 µL (1.5 $x10^8$ CFU/mL, 0.5 McFarland standard) were inoculated into each well of flat 96-well microplate, containing 50 µL of PE (62.5 to 500 µg/mL) in BHI agar. Gentamicin (12 µg/mL) was used as positive control of bacterial growth inhibition and BHI was used as negative control. Plates were read at 620 nm in a spectrophotometer at 24 and 48 h. The optical density (OD630) was corrected by subtraction of the OD630 nm because of the propolis alone in sterile broth. The MIC was determining using the following criteria: (OD630 untreated bacteria - OD630 nm test concentration)/(OD630 nm untreated bacteria) x 100.

2.12. Statistical analysis

All experiments were conducted in triplicate and results are given as mean ± standard deviation, obtained from at least three independent experiments. Data were submitted to analysis of variance (ANOVA) with Tukey-Kramer multiple comparison test. A principal component analysis (PCA) was applied in order to study the effect of pollen source on all variables that contributed to propolis typification (Software package, SPSS version 19). The level of significance was set at $P<0.05$.

3. Results and Discussion

3.1. Palynological identification

The results of the propolis pollen analysis are shown in Table 1. The most frequent pollen types were *Prosopis velutina* (15-29 %; known as Mezquite) and *Mimosa distachya* var. laxiflora (15-20 %; known as Catclaw) which belong to the Fabaceae families ($P<0.05$), mainly in summer season (M2, M4, C2 and C4) that winter season (M1, M3, C1 and C3), and this fact can be explained by the high pollen production by these plant species during summer and because these

plants are frequently visited by bees (Felger, & Moser, 1971; Warakomska, & Maciejewicz, 1992). In terms of melissopalinology, these samples are considered as bi-floral propolis with a high percentage of Mesquite or Catclaw. The pollen types observed in propolis samples can vary according to the region where they are offered, a factor which depends by the dominant plant families available in the apiary and climate conditions for flowering (Barth, 2004), and the pollen content can be a good marker to indicate the botanical source of which the bee collects the necessary components to produce the propolis (Warakomska, & Maciejewicz, 1992).

3.2. Organoleptic and physicochemical analyses

A descriptive analysis of organoleptic and physicochemical parameters is shown in Table 2. The organoleptic parameters in the propolis samples were found to meet with international propolis specifications (NSO, 2003). Samples tested showed organoleptic attributes that characterize to raw propolis such as appearance (mass), color (brown to green), aroma (resinous), flavor (tasteless or light-bitter), consistency (light-sticky or sticky) and light quantity of visible impurities (vegetables, waxes and bees), which are often an indicative of its botanical origin (Funari, & Ferro, 2006). Similar organoleptic properties such as color and consistency were previously reported for Sonoran propolis collected in different seasons (Valencia et al., 2012)

The results of the physicochemical parameters revealed some differences in propolis from different pollen source ($P<0.05$). Propolis moisture content depends on the environmental (temperature and humidity) and manipulation by beekeepers at the harvest period, and it can vary from each season and year (Seidel, Peyfoon, Watson, & Fearnley, 2008). The moisture content of analyzed samples (%) ranged from 1.1 to 4.6%, which are within the allowed parameters (<8%) according to the international regulations of quality (Vargas-Sánchez, Torrescano-Urrutia, & Sánchez-Escalante, 2013). The lowest moisture content values was obtained in propolis from Mesquite (1.3-1.4%) that Catclaw (1.6-4.6%). Higher moisture content could lead to presence undesirable of bacteria

and fungi (Funari, & Ferro, 2006). Ahs content is a parameter used for the determination of the botanical origin and can indicate possibly adulterations in the samples (Funari, & Ferro, 2006). The highest values of ash content (%) were found in samples (M2, M4, C2 and C4) collected in summer (P<0.05), that are outside the allowed parameters (<5%), which can be associated to the presence of inorganic material found in samples (vegetables, waxes and bee). The wax, resin and mechanical impurities contents showed values (<30%, >35% and <25%, respectively) within the legally established intervals (Vargas-Sánchez, Torrescano-Urrutia, & Sánchez-Escalante, 2013). These values ranged from 26-31%, 33-45%, 16-23%, respectively (P<0.05). In our samples, the values were not similar to those previously reported for Colombian propolis (Martínez, Garcia, Durango, & Gil, 2012), whose corresponding values ranged from 1.7-2.9% (moisture), 0.8-4.8% (ash), 48.3-59.3% (wax), 10.5-35.9% (resin) and 14.4-28.2% (mechanical impurities), which indicate the variability of propolis from different geographical origin. High values of mositure, ash, wax and mechanical impurities content resulting reduced the yields and decreased the quality of propolis, while high resins content increase the presence of phenolic compounds (NSO, 2003; Lozina, Peichoto, Acosta, & Granero, 2010).

3.3. Total phenolic and flavonoid content

Phenolic compounds such as flavonoids may directly contribute to biological activities of propolis (Chaillou and Nazareno, 2009). The obtained results showed that the total phenolic content (TPC) determined by Folin–Ciocalteu method, varied between the propolis from different pollen source (P<0.05), ranged from 179.5-397.0 and 67.8-299.7 mg pinocembrin-galangin equivalent/g, for M1-M4 and C1-C4, respectively (Table 4). The highest TPC values were reported for M4>C2, which indicate that there were significant differences between TPC content obtained for propolis collected in different seasons and pollen source. In addition to the TPC, flavones and flavonols (FF), flavanones and dihydroflavonols (FD), are methods commonly used in the quantification of bioactive substances and correlated with the antioxidant activity (Popova et al.,

2004). The content of FF and FD ranged 27.5-136.4 (M1-M4) and 25.2-200.4 (C1-C4) mg rutin/g; and 113.5-257.9 (M1-M4) and 22.3-268.3 (C1-C4), respectively (Table 4). High total flavonoids content (TFC) values were obtained for propolis of different pollen source collected mainly in summer ($P<0.05$). The results obtained indicate that the high levels of TPC and TFC of PE from Catclaw and Mesquite are an important source of biologically active compounds. In a study performed by Valencia et al. (2012), it was found that propolis collected during summer show the highest values of the three main groups of bioactive substances evaluated (total phenolic, flavones and flavonols, flavanones and dihydroflavonols content). According to LeBlanck et al. (2009), Mesquite and Mimosa pollen displayed the highest antioxidant activity compared to Chenopod, Yucca and Palm pollen types, which is due to the pollen types collected by bees generally shows characteristics amounts of total phenolic compounds associate to its geographical and botanical origin. This situation can explain the observed differences between phenolic composition of Mesquite and Catclaw propolis.

3.4. Phenolic composition

The literature indicate that propolis is characterized by containing more than 300 compounds, include phenolic compounds (flavonoids and phenolic acids), which have been identified as constituents of different plant origin (Farré, Frasquet, & Sánchez 2004; Agüero et al., 2011). Analysis of flavonoids and phenolic acids were performed by HPLC-DAD and results indicate that propolis from *Prosopis* and *Catclaw* displayed similar chemical profiles (Table 3). The compounds gallic acid, cinnamic acid, p-coumaric acid, naringenin, quercetin, luteolin, kaempferol, apigenin, pinocembrin, pinobanksin 3-acetate, CAPE, chrysin, galangin, acacetin and pinostrobin were present in all samples regardless of the pollen source. In addition, the major phenolic compounds identified in the eight PE were the flavonoids pinocembrin (M, 11.5-119.4 mg/g; C, 4.7-101.9 mg/g), naringenin (M, 13.6-44.4 mg/g; C, 7.6-136 mg/g), galangin (M, 5.4-18.5 mg/g; C, 1.2-35.5 mg/g), chrysin (M, 2.2-13.0 mg/g; C, 1.8-11.6

mg/g) and quercetin (M, 3.0-6.7 mg/g; C, 3.0-29.5 mg/g). High concentration values were obtained for propolis collected in summer (P<0.05). The indentified phenolic compounds are in agree with those reported by Vargas-Sánchez et al. (2014), who reported that cinnamic acid, quercetin, luteolin, kaempferol, apigenin and pinocembrin were present in PE. In another study performed by Shi et al. (2012), the chemical profile of Chinese propolis was examined and among 40 compounds were identified. This study confirmed that four flavonoids, chrysin, pinocembrin, galangin and pinobanksin 3-acetate, were the primary constituents. Also, profile of phenolic compounds coincides with reports on the identification of phenolic (naringenin) in *Prosopis* and *Mimosa* pollen (LeBlanck et al., 2009). The chemical constituents in propolis are related to bud and wound exudates, as well as the pollen type collected and modified by bees from various flora (Burdock, 1998; Matos, Alencar, & Santos, 2014). In particular phenolic compounds have been reported to have important antifeedant, antimutagenic, anticarcinogen, antiradical and antibacterial properties and protect plants from UV radiation, among others (Ignat, Volf, & Popa, 2011).

3.5. Ferric reducing antioxidant power

In vitro studies have shown strong antioxidant efficacy of PE (Chaillou, Herrera, & Maidana, 2004; Navarro-Navarro et al., 2012). Ferric reducing antioxidant power (FRAP) assay allow determine the reducing ability of the antioxidants present in PE (Benzie, & Strain,1999), which react with the ferric tripyridyl triazine complex Fe^{3+}-TPZ (Table 4). The FRAP values ranged from 2365.0-7152.0 µg mol Fe(II)/g (M1-M4) and 1018.0-3189.2 µg mol Fe(II)/g (C1-C4). High FRAP values were obtained for samples collected in summer (P<0.05). In agree with our results LeBlanc et al. (2009), evaluated the antioxidant activity of different pollen samples from Sonoran desert bee pollen, and they reported that Mesquite and Catclaw pollen types displayed the highest FRAP activity compared to Yucca, Palm, Terpentine Bush and Chenopod bee pollen, which was correlated with the presence of polyphenolic compounds in the extracts.

3.6. Free-radical scavenging activity

The antioxidant activity by Free-radical scavenging activity test (FRS) of M1-M4 and C1-C4 revealed a higher radical-scavenger capacity of the Mesquite propolis compared to Catclaw propolis (Table 4). The FRS is an important property used to prove the capacity of antioxidant components to act as donators of hydrogen atoms (Sharma, & Bhat, 2009). In particular, Mesquite propolis (M1-M4) evidenced an antioxidant capacity between 39.1-72.3% and Catclaw propolis FRS ranged in 11.5-54.4%. Also, high FRS values were obtained for samples collected in summer (P<0.05). In agreement with our results Navarro-Navarro et al. (2012), studied the antioxidant activity of propolis from different areas of northwestern of Mexico. The result showed that the antioxidant activity ranged from 4.0-35 % at 100 µg/ml. Diverse investigations indicated that many of the compounds such as flavonoids group present in Sonoran propolis extracts contributes to the FRS activity and others biological properties (Hernández et al., 2007; Navarro-Navarro et al., 2012; Valencia et al., 2012; Vargas-Sánchez et al., 2014).

3.7. Antibacterial activity.

The antibacterial activity of PE was measured using the broth microdilution method, which is one of the most commonly used techniques to determine the minimal inhibitory concentration (MIC) of antimicrobial agents, including antibiotics and others substances that kill (bactericidal activity) or inhibit the growth (bacteriostatic activity) of bacteria (Wiegand et al., 2008). Various degrees of inhibition against bacterial strains were shown by PE (Table 5). The results showed that at 24 (period in which the bacteria inoculate are stronger), *S*. *aureus* and *L*. *innocua* (Gram-positive) were more sensitive and *E*. *coli* and *S*. *typhimurium* (Gram-negative bacteria) were more resistant. The antibacterial activity was in the following order: *S*. *aureus* > *L*. *innocua* > *E*. *coli* > *S*. *typhimurium* (P<0.05). High inhibition values were obtained for Mesquite propolis (at 500 µg/mL) collected in summer (M2 and M4) against *S*. *aureus* (70-80%) and *L*. *innocua* (65-75%). The same effect was found for E. coli regardless

pollen source for al samples (40% approximately) and for *S*. *typhimurium* the inhibition oscillate between 24 and 42% for Mesquite propolis, which indicate a dependency of the pollen source.

In agreement with our results several investigations indicate that PE inhibit the growth of pathogenic organism, mainly Gram-positive bacteria (Farré et al., 2004). This was consistent with previous studies on other propolis samples (Navarro-Navarro et al., 2012), that through microdilution method determined that PE have a good to moderate antibacterial activity against *S*. *aureus* and *E*. *coli*, respectively. Some researchers have also reported that phenolic compounds from different geographical and plant sources could inhibit various food-borne pathogens, and the total phenol content was highly correlated with antibacterial activity. For example, Eumkeb et al. (2012), reported that luteolin compound, which is commonly present in PE, had an antimicrobial effect against *E*. *coli* (>200 µg/mL), which is associated to the protein inhibition and peptidoglycan synthesis mechanism. Also, the presence of luteolin and its synergism with other flavonoids can explain the high antimicrobial effect on *E*. *coli*. In another study Flavonoids such as, naringenin, hesperetin, 3'-demethoxysudachiqin, pinocembrin, pinobanksin, pinobanksin 3-O-acetate, xanthomicrol, chrysin, galangin and acacetin; and phenolic acid CAPE have been associated with the antimicrobial activity of Sonoran propolis (Hernández et al., 2007). The chemical composition of propolis from *Prosopis* and *Mimosa* obtained in this study exhibits quantitatively considerable differences ($P<0.05$). According to Silici et al. (2007), propolis associated to resins from *Populus* sp., *Eucalyptus* sp., and *Castanea sativa* plant has exhibited a high antimicrobial activity against Gram-positive bacteria such as Bacillus cereus, Enterococus feacalis, Enterococus faecium, *Listeria monocytogenes* (F1483 and F1462), Listeria innocua, *Listeria welshimeri*, *Listeria seeligeri*, *Staphyloccocus aureus* and *Streptococus pyogenes*.

3.8. Component principal analysis

In order to evaluate the differences among propolis from different pollen source, a multivariate analysis (PCA) was carried out (Figure 1A and B). The principal component 1 (PC1) showed 50.0% of variance and the principal component 2 (PC2) 20.3%; thus an accumulative 70.3% of total variation was explained by the two first principal components. The results showed a separation of the samples analyzed. Propolis from Mesquite were separated from Catclaw samples (Figure 1A), which may be associated with the differences in the organoleptic, physicochemical, antioxidant and antibacterial power of the samples (P<0.05). The loadings graph (Figure 1B) showed how each variable tests contributed to the pollen type discrimination. The antioxidant (FRAP, TPC, FRS, FD, and FF) and antibacterial (*S*. *aureus* inhibition) analyses, as well as color and resin content were the most relevant variables correlated with the samples on PCA, mainly in the propolis samples from Mesquite (P<0.05). This was also summarized in Table 6, which shows strong correlation among the results obtained by the antioxidant and antibacterial methods. The lowest correlations were found in the organoleptic (consistency and flavor) and physicochemical characteristic (moisture, ash, wax and mechanical impurities), which is in agreement with Palomino-García et al. (2010), who indicate that these parameters decrease the biological quality of propolis.

Conclusions

The results obtained in this study demonstrated that all propolis samples showed organoleptic and physicochemical properties according to the international regulations of quality. Mesquite propolis type collected in summer was the most effective for act as an antioxidant and antibacterial (mainly against *S*. *aureus*), which was associated with the high total phenolic and flavonoid content, and with the presence of polyphenolic compounds such as pinocembrin, naringenin, galangin, chrysin and quercetin. The multivariate analysis was very helpful in the differentiation of Propolis from Mesquite and Catclaw pollen source. Further studies are necessary to evaluate other variables

that may contribute to floral characterization, as well as the application of the analysis to propolis from other pollen types.

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References

Agüero, M. B., Gonzalez, M., Lima, B., Svetaz, L., Sánchez, M., Zacchino, S., Feresin, G. E., Schmeda-Hirschmann, G., Palermo, G., Wunderlin, D., & Tapia, A. (2009). Argentinean propolis from Zuccagnia punctata Cav.(Caesalpinieae) exudates: phytochemical characterization and antifungal activity. *Journal of agricultural and food chemistry*, *58*, 194-201.

Agüero, M. B., Svetaz, L., Sánchez, M., Luna, L., Lima, B., López, M. L., Zacchino, S., Palermo, J., Wunderlin, D., Feresin, G. E., & Tapia, A. (2011). Argentinean Andean propolis associated with the medicinal plant Larrea nitida Cav. (Zygophyllaceae). HPLC–MS and GC–MS characterization and antifungal activity. *Food and Chemical Toxicology*, *49*, 1970-1978.

Bankova, V. (2005). Chemical diversity of propolis and the problem of standardization. *Journal of Ethnopharmacology*, *100*, 114–117.

Barth, O. M., Dutra, V. M. L., & Justo, R. L. (1999). Pollen analysis of some samples of propolis from Southern Brazil. *Ciência Rural*, *29*, 663-667.

Barth, O. M. (2004). Melissopalynology in brazil: a review of pollen analysis of honeys, propolis and pollen loads of bees. *Science Agricola*, *61*, 342-350.

Benzie, I. F., & Strain, J. J. (1999). Ferric reducing antioxidant power assay: Direct measurement of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Method Enzymology*, *299*, 15-27.

Burdock, G.A. (1998). Review of the biological properties and toxicity of bee propolis (Propolis). *Food and Chemical Toxicology*, *36*, 347–363.

Carpes, S. T., de Alencar, S. M., Cabral, I. S. R., Oldoni, T. L. C., Mourao, G. B., Haminiuk, C. W. I., da Luz C. F. P., & Masson, M. L. (2013). Polyphenols and palynological origin of bee pollen of Apis mellifera L. from Brazil. Characterization of polyphenols of bee pollen. *CyTA-Journal of Food*, *11*, 150-161.

Chaillou, L. L., Herrera, H. A., & Maidana, J. F. (2004). Estudio del propóleos de Santiago del Estero, Argentina. *Food Science and Technology (Campinas)*, *24*, 11-15.

Eumkeb, G., Siriwong, S., & Thumanu, K. (2012). Synergistic activity of luteolin and amoxicillin combination against amoxicillin-resistant *Escherichia coli* and mode of action. *Journal of Photochemistry and Photobiology B: Biology*, *117*, 247-253.

Farré, R., Frasquet, I., & Sánchez A. (2004). El própolis y la salud. *Ars Pharmaceutica*, 45, 23–43.

Felger, R. S., & Moser, M. B. (1971). Seri use of Mesquite (Prosopis glandulosa var torreyana. *Kiva*, *37*, 53-30.

Funari, C. S., & Ferro, V. O. (2006). Análise de própolis. *Ciência Tecnologia Alimentos*, *26*, 171-178.

Hatano, T., Kagawa, H., Yasuhara, T., & Okuda, T. (1988). Two new flavonoids and other constituents in licorice root: Their relative astringency and radical scavenging effects. *Chemical and Pharmaceutical Bulletin*, *36*, 1090–2097.

Hernández, J., Goycoolea, F. M., Quintero, J., Acosta, A., Castañeda, M., Domínguez, Z., Robles, R., Vázquez-Moreno, L., Velázquez, E. F., Astiazaran, H., Lugo, E., & Velázquez, C. (2007). Sonoran propolis: Chemical composition and antiproliferative activity on cancer cell lines. *Planta Medica*, *73*, 1469-1474.

Ignat, I., Volf, I., & Popa, V. I. (2011). A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry*, *126*, 1821-1835.

Jorgensen, J. H., Turnidge, J. D. and Washington, J. A. (1999). Antibacterial susceptibility tests: dilution and disk diffusion methods. In Manual of Clinical Microbiology ed. Murray, P.R., Jo Baron, E., Pfaller, M.A., Tenover, F.C. and Yolken, R.H. Washington DC: ASM Press.pp. 1526–1543.

LeBlanc, B. W., Davis, O. K., Boue, S., DeLucca, A., & Deeby, T. (2009). Antioxidant activity of Sonoran Desert bee pollen. *Food Chemistry*, *115*, 1299-1305.

Lozina, L. A., Peichoto, M. E., Acosta, O. C., & Granero, G. E. (2010) Estandarización y caracterización organoléptica y fisicoquímica de 15 propóleos Argentinos. *Latin America Journal of Pharmacy*, *29*, 102-110.

Lotfy, M. (2006). Biological activity of bee propolis in health and disease. *Asian Pacific Journal of Cancer Prevention*, *7*, 22-31.

Martínez-Florez, S., González-Gallego, J., Culebras, J. M. y Tunon, M. J. (2002). Los flavonoides: propiedades y acciones antioxidantes. Revision. *Nutricion Hospitalaria*, *8*, 271-278.

Martínez, J., Garcia, C., Durango, D., & Gil, J. (2012). Caracterización de propóleos provenientes del municipio de Caldas obtenido por dos métodos de recolección; Characterization of propolis from municipality of Caldas obtained through two collection methods. *Revista MVZ Córdoba*, *17*, 2861-2869.

Matos, V.R., Alencar, S. M., Santos, F. A. R. (2014). Pollen types and levels of total phenolic compounds in propolis produced by *Apis mellifera* L. (Apidae) in an area of the Semiarid Region of Bahia, Brazil. *Anais da Academia Brasileira de Ciência, 86*, 407-418.

Medana, C., Carbone, F., Aigotti, R., & Appendino, G. (2008). Selective analysis of phenolic compounds in propolis by HPLC-MS/MS. *Phytochemical Analysis*, 19, 32–39.

Moreira, L., Dias, L. G., Pereira, J. A., & Estevinho, L. (2008). Antioxidant properties, total phenols and pollen analysis of propolis samples from Portugal. *Food and Chemical Toxicology*, *46*, 3482-3485.

Navarro-Navarro, M., Lugo-Sepúlveda, R. E., García-Moraga, M. C., de la Rosa-López, R., Robles-Zepeda, R. E., Ruiz-Bustos, E., &Velázquez-Contreras, C. (2012). Antibacterial and antioxidant activities of propolis methanolic extracts from Magdalena de Kino and Sonoyta, Sonora. *Biotecnia*, *14*, 9-15.

NSO. (2003). Calidad del propóleos crudo. Norma Salvadoreña 65.19.02:03. NSO 1-12.

Palomino-García, L. R., Martínez-Galán, J. P., García-Pajón, C. M., Gil-González, J. H., & Durango-Restrepo, D. L. (2010). Physicochemical characterization and antimicrobial activity of propolis from municipality of La Union (Antioquia, Colombia). *Revista Facultad Nacional de Agronomía Medellín*, *63*, 5373-5383.

Popova, M., Bankova, V., Butovska, D., Petkov, V., Nikolova-Damyanova, B., Sabatini, A. G., Marcazzan, G. L., & Bogdanov, S. (2004). Validated methods for the quantification of biologically active constituents of poplar-type propolis. *Phytochemical Analysis*, *15*, 235-240.

Popova, M., Silici, S., Kaftanoglu, O., & Bankova, V. (2005). Antibacterial activity of Turkish propolis and its qualitative and quantitative chemical composition. *Phytomedicine*, *12*, 221–228.

Seidel, V., Peyfoon, E., Watson, D. G., & Fearnley, J. (2008). Comparative study of the antibacterial activity of propolis from different geographical and climate zones. *Phytotherapy Research*, *22*, 1256-1263.

Sharma, O. P., & Bhat, T. K. (2009). DPPH antioxidant assay revisited. *Food chemistry*, *113*(4), 1202-1205.

SPSS Statistics. (2012). Statistical Package for the Social Sciences. Chicago, IL, USA.

Tran, V. H., Duke, R. K., Abu-Mellal, A., & Duke, C. C. (2012). Propolis with high flavonoid content collected by honey bees from *Acacia paradoxa*. *Phytochemistry*, *81*, 126-132.

Trusheva, B., Trunkova, D., & Bankova, V. (2007). Different extraction methods of biologically active components from propolis: a preliminary study. *Chemistry Central Journal*, *1*, 13.

Valença, I., Morais-Santos, F., Miranda-Gonçalves, V., Ferreira, AM., Almeida-Aguiar, C., & Baltazar, F. (2013). Portuguese propolis disturbs glycolytic metabolism of human colorectal cancer in vitro. *BMC complementary and alternative medicine*, *13*, 184.

Valencia, D., Alday, E., Robles-Zepeda, R., Garibay-Escobar, A., Gálvez-Ruiz, J. C., Salas-Reyes, M., Jiménez-Estrada, M., Velázquez-Contreras, E., Hernández, J., & Velázquez. C. (2012). Seasonal effect on chemical composition and biological activities of Sonoran propolis. *Food Chemistry*, *1*3, 645-651.

Vargas-Sánchez, R. D., Torrescano-Urrutia, G. R., & Sánchez-Escalante, A. (2013). El propóleos: conservador potencial para la industria alimentaria. *Interciencia*, *38*, 705-711.

Vargas-Sánchez, R. D., Torrescano-Urrutia, G. R., Acedo-Félix, E., Carvajal-Millán, E., González-Córdova, A. F., Vallejo-Galland, B., Torres-Llanez, M. J., Sánchez-Escalante, A. (2014). Antioxidant and antimicrobial activity of commercial propolis extract in beef patties. *Journal of food science*, *79*, C1499-C1504.

Warakomska, Z., & Maciejewicz, W. (1992). Microscopic analyisis of propolis from Polish regions. *Apidologie*, *23*, 277-283.

Wiegand, I., Hilpert, K.,; Hancock, & R. E. W. (2008).Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*, *3*, 163-175.

Table 1
Pollen characteristic of propolis samples

Production year	Season	Pollen contents	Propolis type	Sample code
2012	Winter	>15 % *Prosopis valutina*	Mesquite	M1
2012	Summer	>23 % *Prosopis valutina*	Mesquite	M2
2013	Winter	>17 % *Prosopis valutina*	Mesquite	M3
2013	Summer	>29 % *Prosopis valutina*	Mesquite	M4
2012	Winter	>15 % *Mimosa distachya*	Catclaw	C1
2012	Summer	>20 % *Mimosa distachya*	Catclaw	C2
2013	Winter	>14 % *Mimosa distachya*	Catclaw	C3
2013	Summer	>16 % *Mimosa distachya*	Catclaw	C4

Table 2
Organoleptic and physicochemical characteristic of propolis from different pollen source

Propolis type	Organoleptic aspects					
	Appearance	Color	Aroma	Flavor	Consistency	Visible impurities
M1	Mass	Light B-G	Resinous	Tasteless	Sticky	[V] , [W]
M2	Mass	Brown	Resinous	Tasteless	Light-sticky	[V] , [B] , [P]
M3	Mass	Light B-G	Resinous	Light-bitter	Sticky	[V] , [W]
M4	Mass	Brown	Resinous	Tasteless	Light-sticky	[V] , [W]
C1	Mass	Light B-G	Resinous	Tasteless	Sticky	[V] , [W]
C2	Mass	Brown	Resinous	Tasteless	Light-sticky	[V] , [W] , [B]
C3	Mass	Light B-G	Resinous	Light-bitter	Sticky	[V] , [W]
C4	Mass	Brown	Resinous	Tasteless	Light-sticky	[V] , [P]

Propolis type	collected (g)	Physicochemical aspects (%)				
		Moisture	Ash	Wax	Resin	Mechanical impurities
M1	77.9	1.4 ± 0.3[a]	9.7 ± 0.7[b]	26.2 ± 1.8[a]	43.9 ± 3.3[a]	17.8 ± 0.3[a]
M2	177.8	1.3 ± 0.4[a]	6.7 ± 1.0[a]	28.0 ± 2.7[a]	43.7 ± 1.1[a]	15.7 ± 1.6[a]
M3	63.3	1.2 ± 0.2[a]	6.4 ± 1.3[a]	27.3 ± 2.4[a]	47.0 ± 1.0[a]	16.3 ± 1.4[a]
M4	117.1	1.1 ± 0.3[a]	8.5 ± 0.3[b]	26.0 ± 0.4[a]	47.1 ± 1.2[a]	15.0 ± 1.7[a]
C1	153.8	4.6 ± 0.3[b]	7.4 ± 1.2[b]	27.6 ± 2.4[a]	33.8 ± 2.0[a]	23.5 ± 1.8[b]
C2	54.0	1.6 ± 0.6[a]	2.7 ± 0.6[a]	31.4 ± 0.9[b]	45.5 ± 2.9[b]	17.3 ± 2.7[a]
C3	189.6	4.2 ± 0.3[b]	7.5 ± 2.0[b]	27.2 ± 1.7[a]	34.7 ± 1.2[a]	23.4 ± 0.9[b]
C4	248.3	1.9 ± 0.1[a]	2.5 ± 1.0[a]	29.0 ± 1.3[a]	44.3 ± 1.1[b]	19.5 ± 2.0[a]

[B-G] Brown-Green, [V] Vegetables, [W] Wax, [B] Bees, [P] Painting. All values represent mean of triplicate determination ± standard deviations. Different superscripts (a–b) within the same year of collection and different season differ significantly (P<0.05).

Table 3
Chemical composition of propolis extracts from different pollen source

Compund	Rt	Concentration mg/g							
		M1	M2	M3	M4	C1	C2	C3	C4
Galic acid	1.9	+	+	+	+	+	+	+	+
Chlorogenic acid	2.6	-	-	-	-	-	-	-	-
Cinnamic acid	3.4	1.7^{a}	1.9^{a}	3.0^{b}	2.2^{a}	2.1^{a}	2.2^{a}	3.5^{b}	1.6^{a}
p-coumaric acid	7.8	3.2^{a}	2.9^{a}	0.5^{a}	0.9^{a}	0.1^{a}	1.0^{b}	0.6^{b}	0.1^{a}
Ferulic acid	8.7	-	-	-	-	-	-	-	-
Trans-3-hidroxycinnamic acid	9.6	-	-	-	-	-	-	-	-
Hesperidin	15.1	-	-	-	-	-	-	-	-
Rutin	16.6	-	-	-	-	-	-	-	-
Rosmarinic acid	17.2	-	-	-	-	-	-	-	-
Myricetin	22.2	-	-	-	-	-	-	-	-
Naringenin	27.3	44.4^{a}	54.4^{b}	13.6^{a}	26.8^{b}	7.6^{a}	136^{b}	36.2^{a}	36.9^{a}
Quercetin	30.8	3.0^{a}	6.7^{b}	3.2^{a}	4.9^{a}	0.0^{a}	29.5^{b}	3.0^{a}	3.6^{a}
Hesperetin	31.9	-	-	-	-	-	-	-	-
Genistein	32.5	-	-	-	-	-	-	-	-
Luteolin	36.4	0.2^{a}	3.6^{b}	0.1^{a}	0.5^{a}	0.0^{a}	2.9^{b}	0.8^{a}	0.8^{a}
Kaempferol	37.2	0.1^{a}	0.8^{b}	0.2^{a}	0.2^{a}	0.0^{a}	0.1^{a}	0.0^{a}	0.0^{a}
Apigenin	40.6	4.5^{a}	4.6^{a}	4.5^{a}	4.9^{a}	0.1^{a}	0.2^{a}	1.0^{a}	1.4^{a}
Pinocembrin	44.5	11.5^{a}	119^{b}	25.5^{a}	25.9^{a}	4.7^{a}	102^{b}	18.5^{a}	23.3^{a}
Pinobanksin 3-acetate	45.8	+	+	+	+	+	+	+	+
CAPE	49.0	+	+	+	+	+	+	+	+
Chrysin	51.4	4.0^{a}	13.0^{b}	2.2^{a}	2.3^{a}	1.8^{a}	11.6^{b}	7.5^{a}	7.1^{a}
Galangin	52.4	13.8^{a}	18.5^{b}	5.4^{a}	5.9^{a}	1.2^{a}	35.5^{b}	6.1^{a}	5.3^{a}
Acacetin	57.0	0.3^{a}	0.1^{a}	0.2^{a}	0.2^{a}	0.3^{a}	8.7^{b}	1.1^{a}	1.3^{a}
Pinostrobin	62.9	+	+	+	+	+	+	+	+

Rt, retention time (min); +, compound identified but not quantified; -, compound not identified.
Different superscripts (a–b) within the same year of collection and different season differ significantly ($P<0.05$).

Table 4
Total antioxidant activity of propolis extracts from different pollen source

Propolis type	TPC [A]	FF [B]	FD [C]	FRAP [D]	FRS [E]
M1	179.5 ± 3.3[a]	85.6 ± 3.8[a]	223.8 ± 4.1[a]	2553.2 ± 65.0[a]	41.4 ± 1.4[a]
M2	213.0 ± 2.8[b]	136.4 ± 2.8[b]	257.9 ± 3.3[b]	2883.0 ± 65.4[b]	57.2 ± 1.0[b]
M3	196.9 ± 3.2a	27.5 ± 2.5[a]	113.5 ± 5.1[a]	2365.0 ± 61.0[a]	39.1 ± 1.0[a]
M4	397.0 ± 4.2[b]	46.8 ± 2.8[b]	247.0 ± 2.0[b]	7152.0 ± 32.0[b]	72.3 ± 3.5[b]
C1	67.8 ± 2.4[a]	25.2 ± 0.5[a]	22.3 ± 1.5[a]	1018.0 ± 7.5[a]	11.5 ± 0.5[a]
C2	299.7 ± 1.9[b]	200.4 ± 3.4[b]	268.3 ± 5.7[b]	3189.2 ± 18.1[b]	54.4 ± 1.0[b]
C3	104.9 ± 5.0[a]	47.1 ± 2.1[a]	37.4 ± 3.1[a]	1578.2 ± 47.0[a]	25.5 ± 1.0[a]
C4	128.9 ± 1.5[b]	58.4 ± 1.2[b]	112.9 ± 2.7[b]	2290.3 ± 87.2[b]	41.4 ± 1.2[b]

[A] Expressed as pinocembrin/galangin equivalent
[B] Expressed as rutin equivalent
[C] Expressed as pinocembrin equivalent
[D] Ferric reducing antioxidant power expressed as µg mol Fe(II)/g of propolis
[E] Free radical scavenging activity expressed as %inhibition.
All values represent mean of triplicate determination ± standard deviations. Different superscripts (a–b) within the same year of collection and different season differ significantly (P<0.05).

Table 5
Antibacterial activity (%) of propolis extracts from different pollen source

Propolis type		Gram (+)								Gram (-)							
		S. aureus				*L. innocua*				*E. coli*				*S. typhimurium*			
		500	250	125	62.5	500	250	125	62.5	500	250	125	62.5	500	250	125	62.5
M1	24h	54	34	35	27	55	55	51	44	43	40	41	37	41	19	8	8
	48h	64	42	40	29	54	49	49	47	45	42	41	40	41	23	10	9
M2	24h	82	75	73	30	66	65	61	60	42	38	37	33	42	12	4	4
	48h	87	78	79	77	63	59	58	48	42	41	38	38	17	12	6	6
M3	24h	33	29	30	18	39	37	36	31	42	42	41	37	41	14	8	5
	48h	40	40	36	34	25	24	22	19	45	43	39	38	0	0	0	0
M4	24h	70	69	64	58	51	45	42	39	40	38	38	37	24	19	14	8
	48h	76	73	64	59	46	46	41	39	46	43	41	39	15	14	1	0
C1	24h	36	36	36	16	49	47	44	40	43	40	41	37	41	19	8	8
	48h	34	31	29	19	45	41	42	30	45	42	41	40	41	23	10	9
C2	24h	35	28	25	24	46	45	43	42	42	38	37	33	42	12	4	4
	48h	45	38	38	36	45	46	43	41	42	41	38	38	17	12	6	6
C3	24h	27	27	2	0	55	55	51	44	42	42	41	37	41	14	8	5
	48h	41	27	18	0	56	53	52	53	45	43	39	38	0	0	0	0
C4	24h	34	32	29	20	48	43	40	40	40	38	38	37	24	19	14	8
	48h	46	45	41	32	44	43	39	39	46	43	41	39	15	14	1	0

Positive control Gentamicin (12 µg/mL) exhibited >97% inhibition against all pathogens.

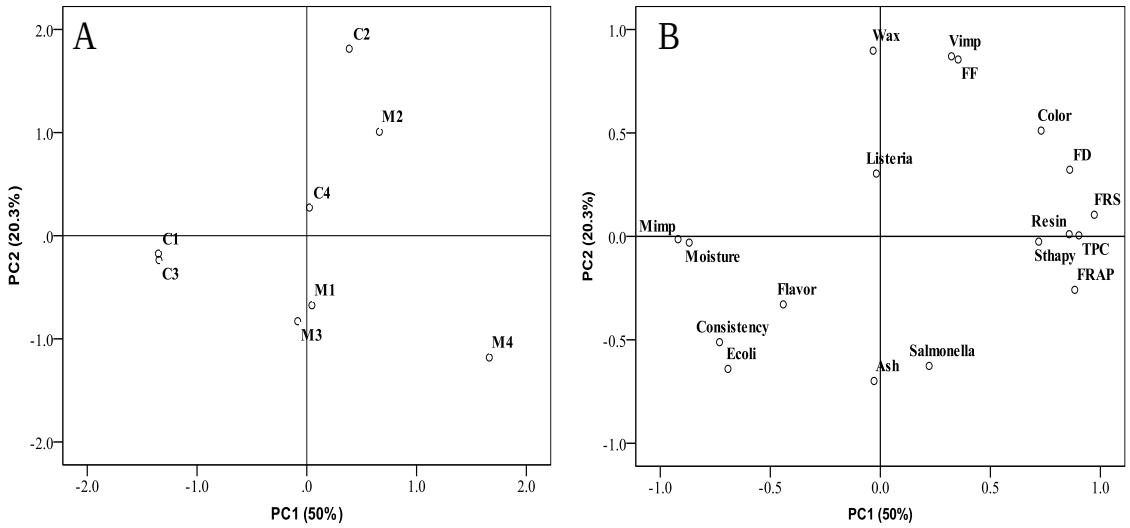

Figure 1
(A) Principal component analysis, in Mesquite (M1 to M4) and Catclaw propolis (C1 to C4); propolis (B) loadings graph.

Table 6
Correlation matrix among the analyzed parameters.

Most	Ash	Wax	Resin	Mimp	TPC	FF	FD	FRAP	FRS	Color	Flavor	Consist	Vimp	Sthapy	List	Ecoli	Salm
1.000																	
.109	1.000	-.894															
-.036	-.894	1.000															
-.977	-.219	.108	1.000														
.961	.007	.022	-.934	1.000													
-.713	.011	.031	.743	-.811	1.000												
-.382	-.441	.714	.330	-.375	.395	1.000											
-.820	-.037	.179	.751	-.849	.806	.720	1.000										
-.601	.171	-.238	.629	-.701	.923	.099	.648	1.000									
-.841	-.077	.046	.824	-.891	.918	.461	.883	.867	1.000								
-.522	-.550	.472	.539	-.545	.608	.562	.647	.575	.761	1.000							
.236	.126	-.210	-.194	.240	-.273	-.416	-.519	-.301	-.344	-.577	1.000						
.522	.550	-.472	-.539	.545	-.608	-.562	-.647	-.575	-.761	-1.00	.577	1.000					
-.313	-.413	.666	.246	-.384	.332	.909	.628	.052	.422	.577	-.333	-.577	1.000				
-.528	.240	-.245	.442	-.671	.515	.148	.605	.576	.624	.525	-.572	-.525	.338	1.000			
.073	.354	-.140	-.273	.014	-.125	.367	.272	-.133	.074	.067	-.195	-.067	.467	.324	1.000		
.515	.562	-.558	-.512	.551	-.592	-.692	-.696	-.495	-.742	-.980	.566	.980	-.728	-.523	-.170	1.000	
-.060	.039	-.411	.154	.008	.108	-.631	-.218	.419	.127	.140	-.081	-.140	-.728	.032	-.510	.059	1.000

CAPÍTULO V

Physicochemical characteristics and biological properties of propolis: CIE color coordinates relationship.

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Running head: Physicochemical and biological properties of propolis may have a relationship with the color.

Physicochemical characteristics and biological properties of propolis: CIE color coordinates relationship.

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ABSTRACT

This experiment was conducted to examine the color relationship of raw propolis collected from different local zones and seasons with the physicochemical and biological properties. In order to estimate this relationship the physicochemical parameters (moisture, ash, was, resin and mechanical impurities content), total phenolic content (TPC), free radical scavenging activity (%FRS), minimum inhibitory concentration (MIC) and color values (L, a, b, C and h) were analyzed. The results obtained by the principal component analysis indicate that the physicochemical characteristics (resin content), antioxidant (TFC and %FRS) and microbiological analyses showed a strong correlation with the color of propolis (mainly h angle).

Keywords: propolis, physicochemical properties, antioxidant activity, antimicrobial activity, color relationship**.**

INTRODUCTION

Honey, royal jelly and propolis, products originating in the beehive, are rich sources of phytochemicals ranging from hydrophilic to lipophilic, such as phenolic acids, aromatic compounds and flavonoids, and they possess a variety of biological properties including antiviral, antifungal, antitumor, anticancer, antioxidant and antibacterial potential. For centuries, dietary herbs, spices and bee product have been traditionally used as food additives throughout the world, especially in Mexico and others countries, not only to improve the sensory characteristics of foods but also to extend their shelf life. Such additives are classified as GRAS (generally recognized as safe).[1, 2, 3]

Propolis is a resinous substance that bees collect from the exudates of plants and which they use to sea holes and crack in the beehive, embalming invaders that bees kills, and protect the colony from diseases. It is mainly composed of resin (50%), wax (30%), essential oils (10%), pollen (5%), and other organic compounds (5%). Among these organic compounds we may find flavonoids in all their forms (flavonoles, flavones, flavonones, dihydroflavonoles, and chalcones). [4, 5, 6] However, its composition varies with different factors, such as source of the exudates, climate, environmental conditions, season, geographical and botanical origin and influences its biological properties, characteristics of propolis such as flavor (bitter or tasteless) and color (yellow, green, brown, red, black).[5, 6, 7]

Information about the color relationships of raw propolis samples with the physicochemical aspects and biological properties is scare. Therefore, the main objectives of this work were to (i) determine the physicochemical characteristics of raw propolis collected from different local zones and seasons in the Norwest of Mexico, (ii) determine the antioxidant activities of propolis extracts by measuring the total phenolic content (TPC) and the free radical scavenging activity (%FRS), and (iii) determine the antimicrobial activity of propolis extracts against food-borne pathogenic bacteria by measuring the minimal inhibitory concentration method. The results of this study will provide information about the

potential of propolis extracts for use in the food industry, classify propolis samples according to their CIE color coordinates and explore the correlation between the physicochemical aspects and biological properties of propolis with the color.

MATERIALS AND METHODS

Propolis samples and extractions of polyphenols

Propolis samples used in this study were collected from 30 hives, which were located in Northwest of Mexico (29.1476 N, -110.1239 O; 632 m). Raw propolis was collected in two main sampling seasons of the year, winter (from December 22 to March 20, 2012 and 2013), and summer (June 22 to September 22, 2012 and 2013). The polyphenolic compounds were extracted from 20 g of raw propolis homogenized with 200 mL of ethanol (at room temperature, 25 °C)) for 3 d for further phenolic compound extraction. Propolis extracts (PEs) were then filtered through Whatman 4 filter paper and concentrated under reduced pressure in a rotary evaporator (BÜCHI R-200, Flawil, Switzerland). Each ethanolic extract was washed three times with 20 mL hexane to remove waxes, and they were stored in the dark at -20 °C until analysis.[8]

Methods

Color measurements

The color measurements were performed using a spectrophotometer (Minolta CM 2600d, Japan). Before each measuring session the colorimeter was calibrated on the CIE color space system using a white tile. The L parameter indicates lightness (L= 0 darkness, L= 100 lightness), a parameter indicates redness (red= +60, green= -60), b parameter indicates yellowness (yellow= +60, blue= -60), C parameter is defined as colorfulness and indicates the perceived strength of a surface color, and h is the degree to which a stimulus can be described as similar to or different from stimuli that are described as red, green, blue and yellow. Fifteen color measurements were performed on the surface of

each sample (raw propolis, 25 °C) with illuminant D65 (Daylight) to 10° angle observer (model CM 2600d, Konica Minolta Inc., Tokyo, Japan).[9]

Physicochemical analysis

The physicochemical analyses was performed to verify the propolis quality.[10, 11]. In moisture and ash content, propolis samples (5 g) were dried in an oven at 100 °C/8 h and incinerate in a muffle at 550 °C/3 h, respectively. The waxes of dry propolis samples (5 g) were extracted under reflux with petroleum ether. After remove the waxes, the sample was placed in an oven at 100 °C/3 h and cooled until constant weight. In mechanical impurities determination, propolis samples were homogenized with n-hexane and ethanol at 40 °C/72 h. The residue was filtered and dried (100 °C/3 h) until constant weight. The resins of dried propolis were extracted under reflux with ethanol and later the solvent was concentrated under reduced pressure in a rotary evaporator. The results were determined gravimetrically.

Total phenolic content

Total phenolic content (TPC) of PEs was determined by the Folin-Ciocalteu method slightly modified.[12] PEs were diluted at different concentrations (62.5, 125 and 250 µg/mL). Then 100 µL of each PEs was mixed with 250 µL Folin-Ciocalteau reagent (1 N), and the reaction was neutralized with 0.750 µL of Na_2CO_3 (7 %, w/v) after 8 min of rest. The sample was homogenized and incubated for 30 min in the dark at 25 °C. The absorbance was measured at 765 nm using a spectrophotometer (model 336001, Spectronic Genesys 5, Thermo Electron Corporation, Madison, WI). Gallic acid was used as the standard for the calibration curve, and results are expressed as gallic acid equivalents (GAE)/g of sample.

Free radical scavenging activity

Free radical scavenging activity (%FRS) was determined by use of the stable 1,1-diphenyl-1-picrylhydrazyl (DPPH)-free radical method slightly modified.[13]

Test tubes with 500 µL of PEs (62.5, 125 and 250 µg/mL) and an equal volume of DPPH solution (300 µmol) were thoroughly mixed by vortex and stored in the dark for 30 min (25 °C). The absorbance was measured at 517 nm in a spectrophotometer (model 336001, Spectronic Genesys 5, Thermo Electron Corporation, Madison, WI). Ascorbic acid (Asc ac, 200 µg/mL) was used as antioxidant standard and DPPH with ethanol as reaction blank. Results were expressed as a percentage decrease in the absorbance with respect to the control values. Inhibition percentage was calculated using the next formula:

FRS (%) = [1 - Abs (S) / Abs (0)] × 100

Where, Abs (0) is the absorbance of the control at t= 0 min; and Abs (S) is the absorbance of the antioxidant at t= 30 min.

The DPPH assay, which has wide spread use in free radical scavenging assessment, is based on reaction between the free DPPH radical and molecules that can donate hydrogen atoms (such as most antioxidants). As a result, a stable non-radical form of the DPPH is obtained, with simultaneous change of the violet color to pale yellow due to the picryl group present in solution.

Minimum inhibitory concentration

The antimicrobial effect of PEs (MIC) was tested against Gram-negative bacteria (*Salmonella* spp and *Escherichea coli* 0157:H7) and Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538P and *Listeria monocytogenes* ATCC 7644) strains by the agar-well diffusion method.[14] Inoculum was prepared using fresh cultures of bacteria strains cultured on nutrient. A loop full of bacteria culture was inoculated into a nutrient broth medium (BHI agar, Difco) and incubated for 24 h at 37 °C. Cell suspension (McFarland turbidity standard 0.5, approximately $1.5x10^8$ colonyforming units CFU/ml) were introduced into nutrient agar plates (Muller-Hilton agar, Difco) and spread thinly on the plates using a glass spreader. Wells (3 mm Ø) were pinched in the plates using a sterile stainless stell borer. The wells were filled with 40 µL of PEs (62.5, 125 and 250 µg/mL). The plates were incubated for 24 h at 37 °C under aerobic conditions. The diameters of the inhibitory zones were measured in millimeters.

Statistical analysis

All experiments were conducted in triplicate and results are represented as mean±standard deviation, obtained from at least three independent experiments. The data analyses were performed using SPSS for Windows Version 21.0 (Statistical Program for Social Sciences, SPSS Inc., Chicago, IL, USA). Data were analyzed using an analysis of variance (ANOVA, GLM) with a post-hoc determination using Tukey's test and a principal component analysis (PCA), in order to study the correlation between all the variables. The level of significance was set at $P<0.05$.

RESULTS AND DISCUSSION

Color of raw propolis

Color coordinates (L, a, b, C and h) of different propolis samples are shown in Table 1. Color is a subjective psycho-physical characteristic as it exists only in the observer's eyes and brain. Currently, color is measured in terms of CIE L, a, b values, Chroma and hue angle. The L, a, b, or CIELab, color space is an international standard for color measurement, adopted by the Commission International d'Eclairage (CIE) in 1976.[15] Propolis color varies from light yellow to brown dark, depending on its botanical source and the time of harvest and is primarily correlated with product quality. [6,7] The results indicate that L and h values showed differences between propolis samples collected in winter (P1 and P3) compared to summer (P2 and P4; $P < 0.05$). The highest values of L value were found in the samples P1 and P3 (L>30), while samples P2 and P4 showed the highest h value. Differences in the parameters a, b, C and h were inconsistent when comparing both seasons collections ($P > 0.05$).

Physicochemical characteristics

The results of the physicochemical parameters revealed differences in propolis from different zone and collect season ($P < 0.05$). The lowest moisture values (1.3-1.5%) were obtained in propolis collected in summer (P2 and P4), which is associated with high temperatures, humidity of the environment and

season of collection.[16] The highest values of ash content were found in propolis samples collected in winter ($P < 0.05$), which is associated with high content of mechanical impurities.[17] The wax content and mechanical impurities showed values within the legally established intervals.[17] High values of wax and mechanical impurities content decreased the quality of propolis.[10, 17]

Antioxidant activity of propolis extracts

TPC of PEs were measured using Folin-Ciocalteu colorimetric method (Fig. 1). The TPC values in summer of PEs ranged from 201-211 mg GAE/g of propolis (at 250 µg/mL) had considerably higher TPC values ($P < 0.05$) compared to winter PEs (154-179, at 250 µg/mL), which is in agree with previous studies.[6] Differences were found in extracts collected at the same time of collection ($P < 0.05$) regardless of the sampling area and concentration evaluated. Farré et al. [7] suggested that TPC in PEs is an important parameter that establishing the biological quality of propolis.

The %FRS assay was based on the measurement of the loss of the deep violet color of the DPPH radical at 517 nm after reaction with an antioxidant compound (Fig. 1). It is rapid test widely used in antioxidant screening to provide information on antioxidant or antioxidant mixture capability in preventing reactive radical species from reaching biomolecules, such as lipoproteins (LPS) or polyunsaturated fatty acids (PUFA), in biological and food systems. The proton-radical scavenging action has been known as an important antioxidant mechanism. DPPH was used to determine the proton-radical scavenging action of PEs. All PEs were potently active and exhibited strong and concentration-dependent DPPH free radical scavenging activity. The free radical scavenging activity values in summer PEs ranged from 83-85% (at 250 µg/mL) had considerably higher values ($P < 0.05$) compared to winter PEs 61-83% (at 250 µg/mL), which is in agree with Valencia et al.[6] Consistently, Chen et al.[18] also found that extracts from propolis collected in summer had significantly higher proton-radical scavenging than PEs collected in summer. Regarding total phenolic content and free radical scavenging activity of PEs significant

correlations were observed (Pearson's correlation coefficients r=0.90), the results suggested a relationship between antioxidant activity and total phenolic content and imply that extracts from propolis may be useful for preventing radical-related food deterioration.

Antimicrobial effect of propolis extracts

Propolis extracts showed different degrees of inhibition against the four phatogenic bacteria (Table 2). The four extracts of propolis tested, exhibited activity against *S. typhimurium*, *L. monocytogenes* and *S. aureus* ($P < 0.05$). PEs did not show effect on the growth of *E. coli* strains at any of the tested concentrations ($P > 0.05$). The highest inhibitory effect was showed in PEs collected in summer (P2 and P4) compared to winter propolis samples (P1 and P3), which is in agree with Chen et al.[18] These results indicated that PEs were promising antimicrobials, notably the activity againts *S. aureus*> *L. monocytogenes*> *S. typhimurium*. Some studies report that phenolic compounds from PEs could inhibit various food-borne pathogens, and the total phenolic content was highly correlated with antibacterial activity.[7, 19] The antimicrobial activity of PEs may involve multiple mode of action: degrade the cell wall, disrupt change fatty acid and phospholipid constituents, influence the synthesis of DNA and RNA. [20]

Relationship between color, physiochemical and biological properties

In order to evaluate the differences among propolis from collected from different zones and season, a multivariate analysis (Principal component analysis, PCA) was carried out (Fig. 2). The principal component 1 (PC1) showed 54.6% of variance and the principal component 2 (PC2) 39.4%; thus an accumulative 94% of total variation was explained by the two first principal components. The results showed a visible separation of the samples analyzed, which may be associated with the differences in the color, physicochemical and biological properties of the samples (i.e. each variable tests contributed to the discrimination of samples; $P<0.05$). For example, The antioxidant (FRS and

TPC) and antimicrobial analyses (MIC determination of PEs in *S. aureus, S. typhimurium*, and *L. monocytogenes*), resin content and color (mainly hue angle, h) were the most relevant variables correlated with the samples on PCA, mainly in the propolis samples collected in summer (P2 and P4; $P<0.05$). This was also observed in Table 3, which shows strong correlation among the results obtained by the antioxidant and microbiological methods with the color (mainly h angle). The lowest correlations were found in the physicochemical characteristic (moisture, ash, wax and mechanical impurities), which is in agreement with Palomino-García et al.[21] who indicate that these parameters decrease the biological quality of propolis.

CONCLUSION

The high contents of TPC, make PEs a promising, inexpensive source of biologically active polyphenolic mixtures. The results of this study indicate an interesting antioxidant and antimicrobial activities of PEs, offer possibilities for their application in preventing oxidative deterioration and/or microbial spoilage of food products. This study also showed that color has a significant correlation with the biological properties.

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REFERENCES

1. Viuda-Martos, M.; Ruiz-Navajas, Y.; Fernández-López, J; Pérez-Álvarez, J.A. Functional properties of honey, propolis, and royal jelly. *Journal of Food Science* **2008**, 73, 117–124.

2. Vázquez-Medina, B.; Martínez-Corona, B.; Aliphat-Fernández, M.M.; Aguilar-Contreras, A. Uso y conocimiento de plantas medicinales por hombres y

mujeres en dos localidades indígenas en Coyomeapan, Puebla, México. *Interciencia* **2011**, 36, 493–499.

3. Negi, P.S. Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. *International Journal of Food Microbiology* **2012**, 156, 7–17.

4. Kalogeropoulos, N.; Konteles, S.J.; Troullidou, E.; Mourtzinos, I.; Karathanos, V.T. Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. *Food Chemistry* **2009**, 116, 452–461.

5. Trusheva, B.; Trunkova, D.; Bankova, V. Different extraction methods of biologically active components from propolis: a preliminary study. *Chemistry Central Journal* **2007**, 1, 13.

6. Valencia, D.; Alday, E.; Robles-Zepeda, R.; Garibay-Escobar, A.; Gálvez-Ruiz, J.C.; Salas-Reyes, M.; Jiménez-Estrada, M.; Velázquez-Contreras, E.; Hernández, J.; Velázquez, C. Seasonal effect on chemical composition and biological activities of Sonoran propolis. *Food Chemistry* **2012**, 131, 645–651.

7. Farré, R.; Frasquet, I.; Sánchez, A. El própolis y la salud. *Ars Pharmaceutica* **2004**, 45, 23–43.

8. Velazquez, C.; Navarro, M.; Acosta, A.; Angulo, A.; Domínguez, Z.; Robles, R.; Robles-Zepeda, R.; Lugo, E.; Goycoolea, F.M.; Velázquez, E.F.; Astiazaran, H.; Hernández, J. Antibacterial and free-radical scavenging activities of Sonoran propolis. *Journal of Applied Microbiology* **2007**, 103, 1747–1756.

9. CIE. Recommendations of uniform color spaces-color difference equations psychometric color terms. *Commision International de I´Eclairage*, Paris. **1978**, Supplement No. 2 to CIE Publication No. 15 (E-1.3.1) 1971/(TC-1.3).

10. Lozina, L.A.; Peichoto, M.E.; Acosta, O.C.; Granero, G.E. Estandarización y caracterización organoleptica y fisico-quimica de 15 propoleos Argentinos. *Latin Amererican Journal Pharmacy* **2010**, 29, 102–11.

11. Martínez, G.J.; Garcia, P.C.; Durango, R.D.; Gil, G.G. Caracterización de propóleos provenientes del municipio de Caldas obtenido por dos métodos de recolección. *Revista MVZ Cordoba* **2012**, 17, 2861–2869.

12. Kaur, R.; Arora, S.; Singh, B. Antioxidant activity of the phenol rich fractions of leaves of Chukrasia tabularis A. *Juss Bioresource Technology* 2008, 99, 7692–7698.

13. Geckil, H.; Ates, B.; Durmaz, G.; Erdogan, S.; Yilmaz, I. Antioxidant, free radical scavenging and metal chelating characteristics of propolis. *American Journal of Biochemical and Biotechnology* **2007**, 1, 27–31.

14. Wiegand, I.; Hilpert, K.; Hancock, R.E.W. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature protocols* **2008**, 3, 163–175.

15. Girolami, A.; Napolitano, F.; Faraone, D.; Fraghieri, A. Measurement of meat color using a computer vision system. *Meat Science* **2013**, 93, 111–118.

16. Seidel, V.; Peyfoon, E.; Watson, D.G.; Fearnley, J. Comparative study of the antibacterial activity of propolis from different geographical and climate zones. *Phytotherapy Research* **2008**, 22, 1256–1263.

17. Vargas-Sánchez, R.D.; Torrescano-Urrutia, G.R.; Sánchez-Escalante, A. El propóleos: conservador potencial para la industria alimentaria. *Interciencia* 2013, 38, 705–711.

18. Chen, Y.W.; Wu, S.W.; Ho, K.K.; Lin, S.B.; Huang, C.Y.; Chen, C.N. Characterisation of Taiwanese propolis collected from different locations and seasons. *Journal of the Science of Food and Agriculture* **2008**, 88, 412–419.

19. Silva, J.C.; Rodrigues, S.; Feás, X.; Estevinho, L.M. Antimicrobial activity, phenolic profile and role in the inflammation of propolis. *Food and Chemical Toxicology* **2012**, 50, 1790–1795.

20. Vargas-Sánchez, R.D.; Torrescano-Urrutia, G.R.; Mendoza-Wilson, A.M.; Vallejo-Galland, B.; Acedo-Félix, E.; Sánchez-Escalante, J.J.; Peñalba-Garmendia, M.C.; Sánchez-Escalante A. Mechanisms involved in antioxidant and antibacterial activity of propolis. Biotecnia 2014, 16, 32–37.

21. Palomino-García, L.R.; Martínez-Galán, J.P.; García-Pajón, C.M.; Gil-González, J.H.; Durango-Restrepo, D.L. Physicochemical characterization and antimicrobial activity of propolis from municipality of La Union (Antioquia,

Colombia*). Revista Facultad Nacional Agronomía Medellín* **2010**, 63, 5373–5383.

Table1 Color values and physicochemical characteristics of the different propolis samples.

Zone	**1**		**2**	
Season	**Winter**	**Summer**	**Winter**	**Summer**
CIE color parameter	**P1**	**P2**	**P3**	**P4**
L	29.1±1.0^{bA}	27.8±0.1^{cB}	34.2±0.8^{aA}	28.9±0.0^{bB}
a	2.6±0.3^{aA}	2.1±0.6^{aA}	3.4±0.7^{aA}	3.5±0.7^{aA}
b	4.9±1.0^{bA}	3.6±0.8^{bA}	7.5±1.7^{aA}	9.6±1.1^{aA}
C	5.5±1.1^{bA}	4.0±0.9^{bA}	8.2±1.8^{aA}	9.1±0.0^{aA}
h	61.4±0.7^{cB}	65.2±0.9^{bA}	65.6±2.1^{bB}	71.6±0.1^{aA}
Physicochemical (%)				
Moisture	4.5±1.0^{aA}	1.5±0.4^{bB}	1.6±0.4^{bA}	1.3±0.4^{bA}
Ash	7.2±1.1^{bA}	2.7±0.6^{cB}	10.4±0.5^{aA}	7.1±0.7^{bB}
Waxes	28.6±1.9^{aA}	27.0±2.4^{aA}	28.0±2.5^{aA}	27.0±2.7^{aA}
Resins	35.5±2.9^{cB}	46.8±2.0^{aA}	42.0±2.9^{bB}	45.7±1.1^{aA}
Mechanical impurities	22.3±1.1^{aA}	17±3.0^{bB}	17.4±1.5^{bA}	16.2±0.5^{bA}

Values represent means ± standard deviations; Means with different letters (a-c; A-B) differ significantly ($P<0.05$).

Table 2 Antimicrobial activity of PE at various concentrations in agar media.

		E. coli			*S. typhimurium*		
(µg/mL)	control	250	125	62.5	250	125	62.5
P1	Ø	Ø	Ø	Ø	10.0 ± 1.4^{bB}	6.5 ± 0.7^{cB}	Ø
P2	Ø	Ø	Ø	Ø	13.5 ± 0.7^{aA}	8.5 ± 0.7^{aA}	Ø
P3	Ø	Ø	Ø	Ø	9.5 ± 0.7^{bB}	6.5 ± 0.7^{cA}	Ø
P4	Ø	Ø	Ø	Ø	11.5 ± 0.0^{aA}	7.0 ± 0.7^{bcA}	Ø
		L. monocytogenes			***S. aureus***		
(µg/mL)	control	250	250	62.5	250	125	62.5
P1	Ø	15.5 ± 0.7^{aA}	8.0 ± 0.0^{aA}	Ø	18.0 ± 0.0^{bB}	9.0 ± 0.0^{aB}	Ø
P2	Ø	12.5 ± 0.7^{bcB}	7.0 ± 0.0^{bB}	Ø	20.0 ± 1.4^{aA}	9.0 ± 0.0^{aA}	Ø
P3	Ø	13.5 ± 0.7^{bA}	7.0 ± 0.0^{aA}	Ø	17.5 ± 0.7^{bA}	9.0 ± 0.0^{aB}	Ø
P4	Ø	11.5 ± 0.7^{cB}	7.0 ± 0.0^{aA}	Ø	18.5 ± 0.7^{bA}	8.0 ± 0.0^{bA}	Ø

Diameter of inhibition zone (mm); Ø no inhibition (0-5 mm); Ethanol (control) was used as control; Values represent means ± standard deviations of inhibition zones; Means with different letters (a-b; A-B) differ significantly ($P<0.05$).

Table 3 Correlation matrix for the color, physicochemical and biological properties parameters of propolis

	L	a	b	C	h	Most	Ash	Wax	Resin	M.imp	TPC	FRS	S.typ	L.mon	S.aur
L	1.000														
a	0.539	1.000													
b	0.375	0.930	1.000												
C	0.527	0.937	0.984	1.000											
h	-0.054	0.804	0.786	0.687	1.000										
Most	-0.158	-0.661	-0.411	-0.364	-0.770	1.000									
Ash	0.857	0.599	0.626	0.749	0.045	0.101	1.000								
Wax	0.292	-0.411	-0.247	-0.133	-0.780	0.898	0.471	1.000							
Resin	-0.201	0.409	0.199	0.103	0.727	-0.932	-0.445	-0.990	1.000						
M.imp	-0.093	-0.675	-0.454	-0.392	-0.824	0.995	0.123	0.923	-0.944	1.000					
TPC	-0.593	0.218	0.164	0.009	0.733	-0.693	-0.645	-0.937	0.886	-0.744	1.000				
FRS	-0.163	0.530	0.351	0.252	0.828	-0.946	-0.342	-0.990	0.987	-0.967	0.889	1.000			
S.typ	-0.743	-0.289	-0.384	-0.518	0.265	-0.440	-0.936	-0.751	0.731	-0.464	0.848	0.651	1.000		
L.mon	-0.813	-0.398	-0.459	-0.595	0.174	-0.317	-0.973	-0.663	0.635	-0.344	0.798	0.550	0.991	1.000	
S.aur	-0.761	-0.431	-0.524	-0.647	0.110	-0.324	-0.973	-0.645	0.633	-0.341	0.754	0.533	0.987	0.994	1.000

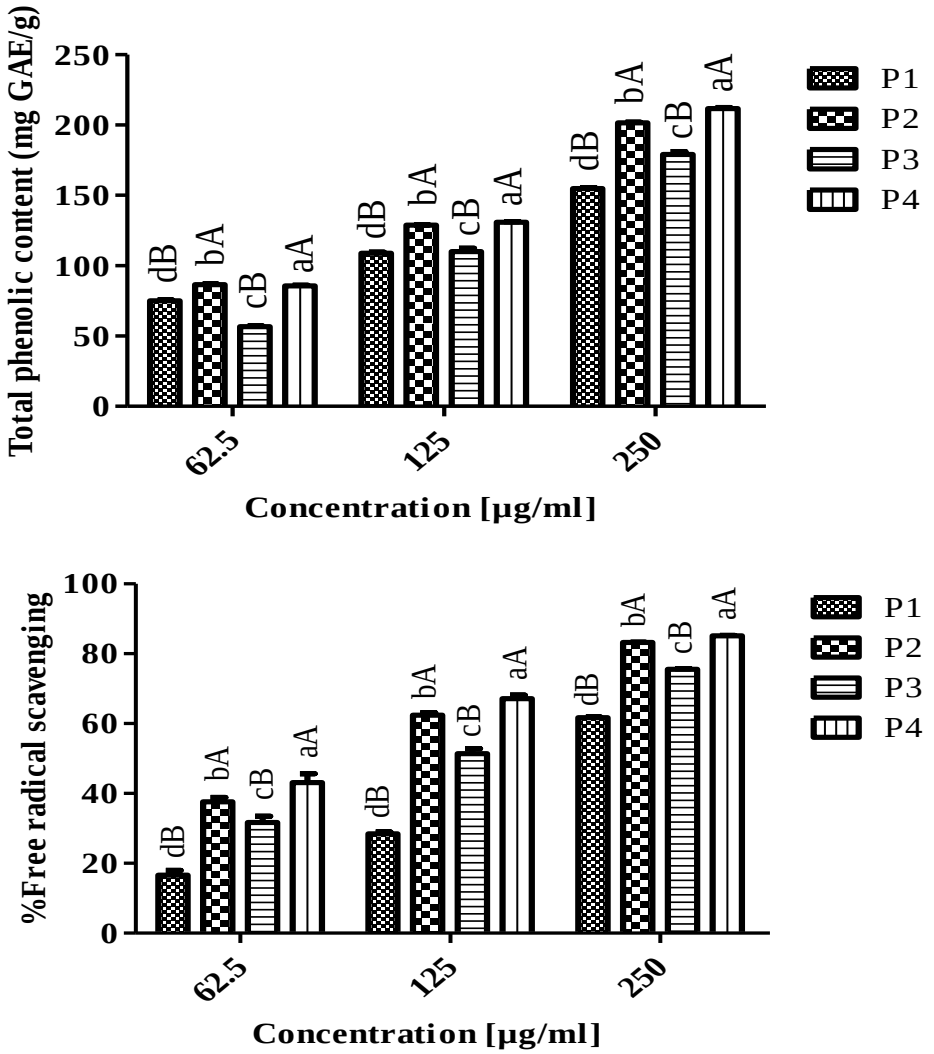

Fig. 1 Antioxidant activity levels in PEs, as determined by the total phenolic content assay (TPC) and free radical scavenging assay (%FRS); Ascorbic acid (Asc ac, 200 µg/mL) was used as control; Values represent means ± standard deviations; Means with different letters ($^{a\text{-}c;\ A\text{-}B}$) differ significantly ($P$<0.05).

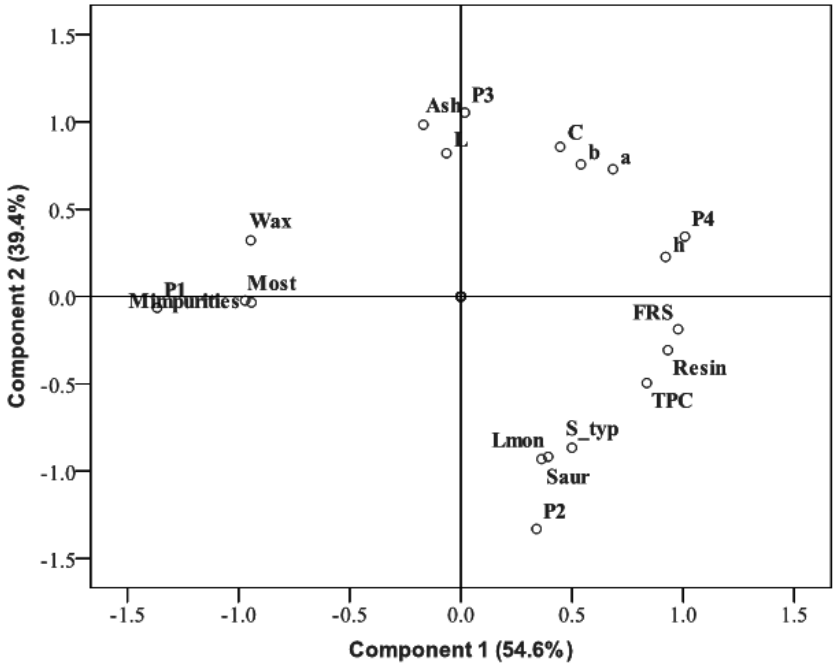

Fig. 2 Principal component analysis of all measured parameters.

CAPÍTULO VI

Mechanisms involved in the antioxidant and antibacterial activity of propolis (2014).
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MECANISMOS INVOLUCRADOS EN LA ACTIVIDAD ANTIOXIDANTE Y ANTIBACTERIANA DEL PROPÓLEOS

MECHANISMS INVOLVED IN ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF PROPOLIS

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RESUMEN

El propóleos es un producto elaborado por las abejas, que se caracteriza por poseer propiedades antioxidantes y antimicrobianas, las cuales son atribuidas a la presencia de compuestos polifenólicos, principalmente flavonoides. Estos compuestos son de bajo peso molecular y en su estructura química contienen un número variable de grupos hidroxilos. Varios investigadores han reportado que los mecanismos antioxidantes propuestos para estos grupos son mediante secuestro de radicales libres, o actuando como substrato para radicales, tales como superóxido e hidroxilo, y quelación de iones metálicos. Mientras que el mecanismo antimicrobiano involucra la inhibición de ácidos nucleicos y degradación de la membrana citoplasmática.

Palabras clave: propóleos, mecanismo antioxidante, antibacteriano, flavonoides.

ABSTRACT

Propolis is a bee product characterized by possesses antioxidant and antimicrobial properties, which are attributed to the presence of polyphenolic compounds, mainly flavonoids. These compounds are low molecular weight and contain in their chemical structure a variable number of hydroxyl groups. Several researchers reported that antioxidant mechanisms proposed for these groups are free radical sequestration, or their acting as substrate for radicals such as superoxide and hydroxyl, and metallic ion chelation. While antimicrobial mechanism involves the inhibition of nucleic acids and degradation of cytoplasmic membrane.

Keywords: propolis, antioxidant mechanism, antibacterial, flavonoids.

INTRODUCCIÓN

El propóleos es una sustancia de composición compleja elaborada por las abejas (*Apis mellifera*), a partir de resinas de ciertas plantas que las abejas modifican por glucólisis con enzimas de las glándulas de la hipofaringe. Posteriormente, la resina parcialmente digerida, es mezclada con cera y polen, y utilizada en la colmena como material de sellado durante el invierno. El propóleos además mantiene un ambiente aséptico en la colmena (Bankova *et al*., 1999; Bankova, 2009). El propóleos es conocido por el hombre desde tiempos remotos, siendo utilizado por los sacerdotes egipcios y más tarde por los griegos, quienes lo denominaron "propóleos", *pro* que significa delante y *polis*, que quiere decir ciudad. De acuerdo a un gran número de estudios, este producto apícola se caracteriza por poseer actividad antiviral, antifúngica, antiinflamatoria, cicatrizante, anestésica, anticancerígena, antioxidante y antibacteriana (Farré *et al*., 2004).

A partir del propóleos se han identificado más de 300 compuestos, de los cuales un alto porcentaje son ácidos fenólicos y sus ésteres, aldehídos aromáticos, cumarinas y flavonoides. En términos de actividad antioxidante y antimicrobiana los principales constituyentes del propóleos son compuestos fenólicos (Kumar *et al*., 2008). Estos compuestos orgánicos se caracterizan por poseer en su estructura molecular al menos un grupo fenol, un anillo aromático unido al menos a un grupo funcional hidroxilo, además algunos son clasificados como metabolitos secundarios de las plantas. Los ácidos fenólicos y flavonoides tienen una variedad muy heterogénea de funciones en las plantas, muchos son productos de defensa ante herbívoros y patógenos; otros proveen soporte mecánico a la planta; otros atraen polinizadores o dispersores de frutos, o actúan como agentes alelopáticos (reducen el crecimiento de plantas competidoras que estén cerca); mientras que algunos de ellos absorben radiación electromagnética en la zona UV-VIS (Kroon y Wi- lliamson, 1999; Martínez-Florez *et al*., 2002), representando una protección natural para las plantas contra la radiación UV del sol, lo cual explica el efecto protector de la oxidación de la piel por ciertos preparados a base de extractos de propóleos; además de la barrera química de defensa contra microorga- nismos (hongos, bacterias y virus) en las colmenas (Cushnie y Lamb, 2005a). Choi *et al*. (2006), evaluaron la actividad anti- oxidante y antimicrobiana *in vitro* de extractos de propóleos, encontrando alta actividad antiradical (DPPH) y un efecto frente a *Staphylococcus aureus* y otros microorganismos Gram positivos, lo cual fue correlacionado con la presencia de flavonoides. Por otro lado, se ha reportado que ciertos ácidos fenólicos derivados del ácido cinámico, que están pre- sentes en los extractos de propóleos, exhiben interesantes propiedades farmacológicas (Kroon y Williamson, 1999). Ac- tualmente, existe mucha evidencia derivada de resultados de investigaciones que demuestran el potencial biológico de los extractos de propóleos. Sin embargo, debido a su compleja

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composición química, muchos de los mecanismos no han sido esclarecidos. Por lo tanto, el objetivo de esta revisión es analizar algunos de los posibles mecanismos por los cuales en base a su composición química, el propóleos ejerce su acción antioxidante y antimicrobiana.

Composición Química del Propóleos

El propóleos es una mezcla compleja de resinas y bálsamos (50-55 %), ceras (25-35 %), aceites volátiles (10%), polen (5 %), minerales y sustancias orgánicas (5 %). En las ceras de este se ha reportado la presencia de ácidos alifáticos (decanóico, dodecanóico, eicosanóico, esteárico, heptadecanóico, hexacosanóico, hidroxiacético, linoleico, málico, nanóico, oleico, palmítico, succínico, tetracosanóico) y algunos ésteres (benzil benzoato, benzil cafeato, etil pal- mitato, cafeato de cinamil, cafeato de fenetil, ftalato y metil palmitato). En aceites volátiles encontramos el α-copaeno, β-cariofileno, aromadendreno, α-humuleno, 9-epi-β-cariofileno, γ-muruleno, δ-amorfeno y óxido cariofileno. En el polen destaca la presencia de proteínas y aminoácidos (ar- ginina y prolina comprenden el 46 %) y azúcares (2-O-gliceril galactosa, ácido galacturónico, ácido glucónico, galactitol, metilglucosa, inositol y xilitol). Además, se pueden encontrar minerales (Al, Ag, Ba, B, Cr, Co, Cu, Sn, Fe, Mg, Mn, Mb, Ni, Pb, Se, Si, Sr, Ti, V y Zn), vitaminas (A, B1, B2, B3 y B6) y sustancias orgánicas. Esta composición es dependiente del origen botánico e influye en las características generales del propóleos, como el color (amarillo, verde, pardo, castaño, rojizo o negro) y sabor (amargo, picante o insípido), principalmente (Abd El Hady y Hegazi, 2002; Peña, 2008).

Compuestos Fenólicos

En la mayoría de los estudios, la actividad antioxidante y antimicrobiana de este producto apícola se atribuye principalmente a los flavonoides: acacetina, apigenina, crisina, galangina, kaempferol, naringenina, pinobanksina, pinocembrina y quercetina (Figura 1). Los flavonoides son compuestos que en su estructura química (básica) tienen un número variable de grupos hidroxilo fenólicos; además, poseen un esqueleto común de difenilpiranos (C6-C3-C6), compuesto por dos anillos de fenilos (A y B) ligados a través de un anillo C de pirano (heterocíclico). Los átomos de carbono en los anillos C y A se numeran del 2 al 8 y los del anillo B desde el 2' al 6'. En función de sus características estructurales se pue- den clasificar en: flavanos (poseen un grupo OH en posición 3 del anillo C), flavonoles (contienen un grupo carbonilo en posición 4 y un grupo OH en posición 3 del anillo C), flavonas (tienen un grupo carbonilo en posición 4 del anillo C y care- cen del grupo hidroxilo en posición C3) y antocianidinas, las cuales tienen unido el grupo OH en posición 3 y un doble enlace entre los carbonos 3 y 4 del anillo C (Havsteen, 2002; Martínez-Florez *et al*., 2002).

Sin embargo, recientemente se ha incrementado el estudio de ácidos fenólicos presentes en extractos de propóleos, entre ellos ácido benzoico, ácido cafeico, ácido ferúlico, ácido cinámico, ácido *p*-cumárico y el éster fenetílico del ácido cafeico (CAPE). Los ácidos fenólicos son divididos en dos grupos, en base a dos marcos de carbono distintivos: el hi- droxibenzoico y las estructuras hidroxicinámicos. Los ácidos hidroxicinámicos o fenilproponides (C_6-C_3) están formados básicamente por un anillo aromático, un grupo alifático y un ácido carboxílico en el extremo. Son denominados hidroxi- cinámicos por poseer un grupo –OH en el anillo aromático, el cual es un grupo muy relacionado con su actividad en sistemas biológicos (Cos *et al*., 2002; Shaidi y Chandrasekara, 2010).

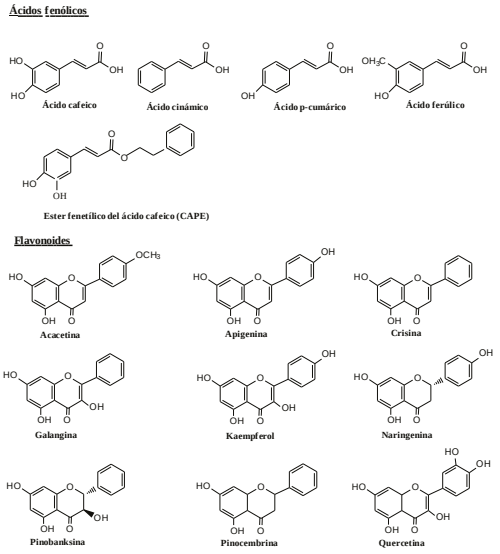

Figura 1. Estructura química de los compuestos con actividad biológica más importantes del propóleos.

Figure 1. Chemical structure of compounds with important biological activities of propolis

Estos compuestos difieren en concentración y actividad biológica, dependiendo del origen botánico del propóleos (Hamasaka *et al*., 2004; Lou *et al*., 2012). Sin embargo, para establecer algún mecanismo de acción de los compuestos presentes en los propóleos, es necesario conocer su estructura y el posible comportamiento, simulando condiciones fisiológicas parecidas a las de organismos vivos.

Actividad Antioxidante del Propóleos

En los últimos años diversas investigaciones han destacado al propóleos como un antioxidante de origen natural, para la prevención y tratamiento de diversas enfermedades de origen oxidativo (Farré *et al*., 2004). Chopra *et al*. (1995), estudiaron el efecto protector del propóleos y el efecto individual de algunos flavonoides como la rutina, en ratas con miocardiopatía, enfermedad producida por el estrés oxi- dativo; Irmak *et al*. (2003), determinaron el efecto del CAPE y el α-tocoferol, en ratas con lesiones cerebrales (isquemia

cerebral), generadas por la liberación de radicales libres. Los resultados mostraron que la administración aguda de CAPE y α-tocoferol suprimió la isquemia inducida por la peroxi- dación lipídica y el daño cerebral, encontrándose que CAPE ofrece una mayor ventaja terapéutica que el α-tocoferol; Moraes *et al.* (2010), evaluaron muestras de propóleos brasi- leños para conocer el efecto antiproliferativo sobre tumores malignos primarios (RC-58T/h/SA#4), derivados de células de cáncer prostático humano. Por otra lado, Li-Chang *et al*. (2003), estudiaron la actividad antiradical DPPH de extractos etanólicos de propóleos (EEP) recolectados en las regiones de Mingchien, Fangliao y Taipie (Taiwán). En este trabajo los EEP (100 µg/mL) mostraron valores superiores a 80 % de acti- vidad antiradical, probando que el propóleos presenta fuerte inhibición de este tipo de células y alta actividad antioxidante en los extractos evaluados.

Inhibición de Radicales Libres y Quelación de Iones Metálicos

Las reacciones oxido-reductoras constituyen una parte esencial del metabolismo aeróbico en los organismos vivos y ocurren principalmente entre moléculas orgánicas. Durante el desarrollo de este tipo de reacciones en sistemas biológicos normales, como la producción de energía, fagocitosis, regulación del crecimiento celular, señalización intercelular y síntesis de compuestos biológicos importantes como el ATP, es común que se formen sustancias reactivas al oxígeno (ROS) a manera de intermediarios: oxígeno (O_2) y peróxido de hidrógeno (H_2O_2). Sin embargo, el organismo puede sufrir ciertos desequilibrios que conducen a la formación de ROS con actividad de radicales libres por la transferencia de elec- trones desapareados, atacando los lípidos de las membranas celulares, modificando proteínas estructurales, así como carbohidratos y ADN (Erkoc *et al*., 2003; Lenhninger *et al.*, 1995), asociándose la formación de este tipo de ROS con la aparición de ciertas enfermedades; por lo que, el organismo a manera de defensa utiliza su sistema endógeno y exógeno de antioxidantes. Este último involucra a los flavonoides y ácidos fenólicos, que son adquiridos por medio de la dieta (Kroon y Williamson, 1999; Martínez-Florez *et al*., 2002). En los alimentos la inhibición de radicales libres está asociada al incremento de la vida de anaquel, ya que muchos de estos productos contienen cierto porcentaje de lípidos, los cuales pueden presentarse en forma saturada, mono-insaturada y poli-insaturadas, siendo los ácidos grasos poli-insaturados los más susceptibles al proceso de oxidación; por lo que para detener o retardar este tipo de reacciones, en las últimas décadas se hace uso de fuentes sintéticas o naturales (Loliger, 1991).

La actividad antioxidante se lleva a cabo mediante una transición redox, a través de la cual la molécula antioxidante libera un átomo de hidrógeno que puede ser captado por un radical libre, o permitiendo la formación de ligandos que faciliten la quelación de iones metálicos (Fe^{2+}, Cu^{2+}, Zn^{2+}) y la interacción con enzimas. Los flavonoides y ácidos fenólicos tienen la propiedad de interceptar y reaccionar con agentes oxidantes como enzimas, metales y radicales libres (Tabla 1). Velázquez *et al*. (2007), evaluaron la actividad antiradical (DPPH) de propóleos Sonorenses de diversos compuestos fenólicos (CAPE, galangina y rutina), encontrando que estos tienen alta afinidad por atrapar radicales libres, en comparación con otros aditivos de origen sintéticos como el butilhidroxitolueno (BHT). Geckil *et al*. (2005), estudiaron las características antioxidantes del propóleos, es decir su capa- cidad para atrapar radicales libres y quelar iones metálicos, así como la capacidad para retardar la peroxidación lipídica, reportando suficiente evidencia científica que sugiere que el alto potencial antioxidante del propóleos, es debido a su rica fuente de compuestos polifenólicos. De acuerdo a lo anterior, se establece que el mecanismo antioxidante más común para flavonoides y ácidos fenólicos presentes en los propóleos, es su capacidad de inhibir radicales libres y de quelación de iones metálicos. Ambos mecanismos son totalmente depen- dientes de la capacidad para donar electrones de los grupos que forman parte de la estructura química del compuesto (Jovanovic *et al*., 1994; Yao *et al.*, 2004). Al respecto aún existe controversia; sin embargo, se plantean tres vías para explicar el mecanismo mediante el cual los compuestos fenólicos (ArOH) inhiben radicales: 1) abstracción del H-átomo (HAT), 2) transferencia secuencial de la pérdida del protón y del electrón (SPLET), y 3) transferencia secuencial de la pérdida del electrón seguido del protón (SET-PT) (Mendoza-Wilson y Glossman-Mitnik, 2004).

Actividad Antibacteriana del Propóleos

Además de la actividad antioxidante, otra propiedad funcional del propóleos es su capacidad antimicrobiana (Tabla 1), una de las primeras propiedades constatadas, lo cual ha sido probado por la existencia de múltiples estudios bacteriológicos *in vivo* e *in vitro*, donde se ha confirmado su acción bacteriostática y bactericida (FAO, 1996; Tosi *et al*., 1996). Velázquez *et al.* (2007), evaluaron la actividad antimi- crobiana de muestras de propóleos colectadas en diferentes áreas del desierto de Sonora (Noroeste de México). Los mi- croorganismos utilizados para este estudio fueron bacterias Gram positivas (*S. aureus*, *E. faecalis* y *L. monocytogenes*) y Gram negativas (*E. coli* y *P. aeruginosa*), utilizando extractos metanólicos de propóleos (400, 200, 100 y 50 µg mL^{-1}). En todas las concentraciones de propóleos evaluadas, no se presentó efecto alguno sobre bacterias Gram negativas. Mientras que en las bacterias Gram positivas, *S. aureus* fue el microorganismo sobre el que se encontró la más alta efecti- vidad. Esta actividad fue correlacionada con la presencia de algunos compuestos fenólicos: acacetina, CAPE, crisina, ga- langina, pinocembrina, pinobanksina y naringenina. Sagdic *et al*. (2007), evaluaron el efecto de un extracto de propóleos (1, 2 y 5 %), sobre la descomposición de jugo de manzana y el crecimiento de bacterias Gram negativas, como *Escherichia coli* y *E. coli* O157:H7, encontrando un efecto inhibitorio a altas concentraciones de propóleos. En estos estudios la actividad antioxidante y antimicrobiana demostró una fuerte dependencia al origen botánico, periodo de colecta, con-

Tabla 1. Actividad antioxidante y antibacteriana (relación estructura actividad) de algunos compuestos polifenólicos.
Table 1. Antioxidant and antibacterial activity (structure-activity relationships) of some polyphenolic compounds.

Compuesto	Actividad antioxidante (comentarios)	Referencias
Apigenina y Naringenina; Kaempferol y Quercetina	Hidroxilación en los grupos 5, 7 y 4´, presentan propiedades antiradicales; hidoxilación en carbono 3, presenta mayor actividad que en los sustituyentes 5, 7 y 4´ hidroxilación.	Das y Pereira, 1990
Ácidos cafeico y ferúlico	La presencia de grupos –CH = CH–COOH en ambos compuestos, pueden ser responsables de la actividad antioxidante; La presencia de grupos donadores de electrones en el anillo de benceno (3-metoxi y en mayor importancia 4-hidroxil), inhibe la formación de radicales libres; el grupo carboxilo con una insaturación adyacente (C-C), provee un sitio para ataques de radicales y posee cierta protección contra la peroxidación lipídica	Kumar-Maurya D, y Asir-Devasagayam TP, 2010; Ergün *et al*., 2011; Kanaski *et al*., 2002
Microorganismo	Actividad antimicrobiana (comentarios)	
S. aureus resistente a metacilina (MRSA)	2´, 4´ o 2´,6´- dihidroxilación (anillo B) y 5, 7 -dihidroxilación del anillo A de la estructura de las flavanonas, presenta mayor actividad anti-MRSA; Sustitución en la posición 6 u 8 con una larga cadena de grupos alifáticos tales como lavandulilo (5-metil-2-isopropenil-hex-4-enil) o geranil (trans-3 ,7-dimetil-2, 6 -octadienil) también presentan actividad antibacteriana; 3, 5, 7-trihidroxiflavona (Galangina), acción por actividad lipofilica.	Tsuchiya *et al*., 1996; Pepeljnjak y Kosalec, 2004.
St. mutans y *St. sobrinus*; *St. species*	Sustitución de 1, 2 o 3 grupos hidroxilos en los grupos 7, 2´, y 4´ del 5-hidroxiflavanonas; Isoflavonas con grupos hidroxilos en posición 5, 2´ y 4´, presentan actividad, sugiriendo que la hidroxilación 2´ es importante para la actividad.	Osawa *et al*., 1992; Sato *et al*., 1996.
Bacterias orales	Hidroxilación en el grupo 2´ del flavonoide es efectiva para su actividad antimicrobiana.	Sato *et al*., 1997
P. vulgaris y *S. aureus*.	Enlaces de hidrógeno de los ácidos nucleicos con el anillo B del flavonoide, son indispensables para inhibir la síntesis de ácidos nucleicos (ADN y ARN)	Mori *et al.*, 1987.
S. aureus, S. epidermis, E. coli, S. typhimurium y *S. maltophilia; St. pneumoniae, B. subtilis, E. coli, S. dysenteriae* y *S. typhimurium; E. faecalis y E. faecalis* mayor resistente a vancomicina	DNA girasa fue inhibida en *E. coli* por los flavonoides quercetina, apigenina y 3, 6, 7, 3´, 4´-pentahidroxiflavona, la enzima se limitó a compuestos con B-hidroxilación del anillo; El ácido p-cumárico, posee alta permeabilidad en la membrana bacteriana e interaciona con los pares de bases del ADN, lo que resulta en una actividad antimicrobiana; Ésteres de ácido ferúlico (6b y 6c),	Hilliard *et al*., 1995; Ohemeng *et al*., 1993; Lou *et al*., 2012; Ergün *et al*., 2011

centración y tipo de compuestos presentes, sin embargo, en los resultados no mencionan los mecanismos que pudieran estar involucrados.

Inhibición de la Síntesis de Ácidos Nucleicos y Degradación de la Membrana Citoplasmática

La amplia gama de funciones de las células en sistemas eucarióticos pueden ser afectadas por los flavonoides y ácidos fenólicos. Sin embargo, es poca la información publicada en referencia a la relación existente entre la estructura de estos compuestos y su actividad antimicrobiana (Tabla 1). Simuth *et al*. (1986), demostraron que los compuestos presentes en el propóleos que absorben en la región UV inhibían el ADN dependiente de la ARN polimerasa de *E. coli* y *Strep- tomyces aureofaciens*. Sin embargo, el mecanismo de acción del propóleos sobre estos microorganismos en aquel entonces parecía ser muy complejo. Mori *et al.* (1987), realizaron un estudio con precursores radiactivos y encontraron que los flavonoides robinetina, miricentina y (-)-epigalocatequina, inhibieron la síntesis de ADN en *P. vulgaris*, mientras que para *S. aureus* se inhibió la síntesis de ARN, aunque la síntesis de proteínas y lípidos también fueron afectadas, pero en menor grado. Posteriormente, Ohemeng *et al*. (1993), evidenciaron la actividad inhibitoria de la estructura de 14 flavonoides contra la ADN girasa (enzima participante en la replicación del ADN) de *E. coli*, S. *epidermis*, *S. aureus, S. typhimurium* y *S. maltophilia.* La ADN girasa de *E. coli* fue inhibida por sólo siete de los compuestos, entre ellos quercetina, apigenina y 3, 6, 7, 3, 4–pentahidroxiflavona. Sin embargo, ésta no siempre fue correlacionada, por lo que sugirieron otros mecanismos de

participación.

Ikigai *et al*. (1993), investigaron el efecto antibacteriano de (-)-galato de epigalocatequina, utilizando un modelo de membrana de liposomas, encontrándose que este compuesto perturbó la bicapa lipídica, alterando la función de barrera y en algunos casos, se redujo el espacio intraliposomal causando la fusión de membranas, y provocando fuga y agregación de material intramembranoso. Mirzoeva *et al*. (1997), demostraron que quercetina y naringenina incrementan la permeabilidad y disipan el potencial de la membrana bacteriana (fuerza motriz de protones), disminuyendo la resistencia bacteriana a los antibióticos. Estos flavonoides también inhibieron la motilidad bacteriana, factor importante en la virulencia. Posteriormente, Takaisi-Kikuni y Schilcher (1994), evaluaron mediante microcalorimetría y microscopía electrónica el modo de acción antibacterial del propóleos y encontraron que, pinocembrina, galangina y CAPE causan bacteriólisis parcial (*Streptoccocus agalactiae*), previniendo la división celular, desorganizando el citoplasma y la pared celular, y por inhibición de síntesis de proteínas y ARN polimerasa.

En estudios más recientes, Cushnie y Lamb (2005b), reportaron que la galangina incrementa la pérdida de potasio en *Staphyloccocus aureus*, degradando la membrana citoplasmática de las bacterias por lisis osmótica. Lou *et al*. (2012), encontraron que el ácido p-cumárico (10-80 µg/mL) inhibe el crecimiento bacteriano, lo cual fue correlacionado con un aumento en la permeabilidad de la membrana bacteriana e inhibición del ADN. Orsi *et al.* (2006), reportaron que los EEP tienen marcados efectos sinérgicos con algunos antibióticos (amoxicilina y cefalaxina) frente *S. typhi*, por su acción sobre la pared celular, resultado que es de gran importancia en la medicina, ya que el costo de los antibióticos puede ser reducido. Eumkeb *et al*. (2012), evaluaron la actividad sinérgica de luteolina y amoxicilina contra *E. coli* resistente a amoxicilina (AREC, por sus siglas en inglés), así como su posible mecanismo de acción; encontrando que existe un efecto sinérgico debido a la combinación del pro- póleos y amoxicilina, al reducir el número de células de AREC. Además, esta combinación alteró la permeabilidad de la membrana, tanto en el interior como el exterior. De acuerdo a estas investigaciones, se puede establecer que algunos de los mecanismos de acción antimicrobiana de los flavonoides y ácidos fenólicos presentes en el propóleos están estrecha- mente relacionados al tipo de compuesto, efecto sinérgico y a su estructura. Sin embargo, también es posible establecer que cada compuesto posee un punto de acción diferente en las bacterias (Cushnie y Lamb, 2005a).

CONCLUSIONES

La capacidad antioxidante y antimicrobiana de los extractos de propóleos se atribuye a la presencia de un alto contenido de compuestos fenólicos, principalmente flavonoides y ácidos fenólicos, y a la posible interacción individual o sinérgica de cada una de las estructuras que los conforman. El mecanismo de acción antioxidante y antimicrobiana del propóleos es muy complejo; en el caso del primero, la principal ruta puede operar a través de la inhibición de radicales libres en etapas tempranas o tardías de la oxidación, mientras que para el segundo mecanismo, pueden ser un conjunto de variables que impidan a la bacteria llevar a cabo su desarrollo normal.

REFERENCIAS

Abd El Hady, F.K. y Hegazi, A.G. 2002. Egyptian propolis; 2 – Chemical composition, antiviral and antimicrobial activity of East Nile Delta propolis. Z. Zeitschrift für Naturforschung. 57: 386-391.

Bankova, V., Boudourova-Krasteva, G., Sforcin, M.J., Frete, X., Kujumgiev, A. y Maimoni-Rodella, R. 1999. Phytochemical evidence for the plant origin of Brazilian propolis from Sao Paulo state. Zeitschrift für Naturforschung. 54: 401-405.

Bankova V. 2009. Review: chemical diversity of propolis makes it a valuable source of new biologically active compounds. Journal of ApiProduct and ApiMedical Science. 1(2): 23–28.

Choi YM, Noh DO, Cho SY, Suh HJ, Kim KM, y Kim JM. 2006. Anti- oxidant and antimicrobial activities of propolis from several regions of Korea. LWT- Food Science and Technology. 39: 756-761.

Chopra, S., Pillai, K.K., Husain, S.Z. y Giri, D.K. 1995. Propolis pro- tects against doxorubicin-induced myocardiopathy in rats. Experimental and Molecular Pathology. 62: 190-198.

Cos, P., Rajan, P., Vedernikova, I., Calomme, M., Pieters, L., Vliet- inck, A.J., Augustyns, K., Haemers, A. y Berghe, D.V. 2002. In vitro antioxidant profile of phenolic acid derivatives. Free Radical Research. 36:711–716.

Cushnie, T.P.T. y Lamb, A.J. 2005a. Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents. 26:343-356.

Cushnie, T.P. y Lamb, A.J. 2005b. Detection of galangin-induced cytoplasmic membrane damage in *Staphylococcus aureus* by measuring potassium loss. Journal of Ethnopharmacology. 101: 243–248.

Das, N.P. y Pereira, T.A. 1990. Effects of flavonoids on thermal autoxidation of palm oil: structure-activity relationships. Journal of the American Oil Chemists' Society. 76(4): 255-258.

Ergün, B.Ç., Çoban, T., Onurdag, F.K. y Banoglu, E. 2011. Synthe- sis, Antioxidant and Antimicrobial Evaluation of Simple Aro- matic Esters of Ferulic Acid. Archives of Pharmacal Research. 34(8): 1251-1261.

Erkoc, S., Erkoc, F. y Keskin, N. 2003. Theoretical investigation of quercetin and its radicals isomers. Journal of Molecular Structure. TEOCHEM. 631:141-146.

Eumkeb, G., Siriwong, S. y Thumanu, K. 2012. Synergistic activity of luteolin and amoxicilin combination against resistant *Escherichia coli* and mode of action. Journal of Photochemis- try and Photobiology. 117: 247-253.

FAO. 1996. Value-added products from beekeeping. Food and Agriculture Organization of the United Nations. Bulletin no. 124. Roma.

Farré, R., Frasquet, I., y Sánchez, A. 2004. El própolis y la salud. Ars Pharmaceutica. 45(1): 23-43.

Geckil, H., Ates, B., Durmaz, G., Erdogan, S. y Yilmaz, I. 2005. Antioxidant, free radical scavenging and metal chelating characteristics of propolis. American Journal of Biochemis-

try and Biotechnology. 1(1): 27-31.

Kumazawa, S., Fujimoto, T. y Nakayama, T. 2004. Antioxidant activity and constituents of propolis collected in various areas of Japan. Food Science and Technology Research. 10 (1): 86–92.

Havsteen, B.H. 2002. The biochemistry and medical significance of flavonoids. Pharmacology and Therapeutics. 96: 67-202.

Hilliard, J.J., Krause, H.M. y Bernstein, J.I. 1995. A comparison of active site binding of 4-quinolones and novel flavone gyrase inhibitors to DNA gyrase. Advances in Experimental Medi- cine and Biology. 390:59–69.

Ikigai, H., Nakae, T., Hara, Y. y Shimamura, T. 1993. Bactericidal catechins damage the lipid bilayer. Biochemical and Bio- physica Acta. 1147: 132–6.

Irmak, M.K., Fadillioglu, E. y Sogut, S. 2003. Effects of caffeic acid phenethyl ester and alpha-tocopherol on reperfusion injury in rat brain. Cell Biochemistry and Function. 21: 283-289.

Jovanovic, S.V., Steenken, S., Tosic, M., Marjanovic, B. y Simic, M.G. 1994. Flavonoids as antioxidants. Journal of American Chemistry Society. 116: 4846-4851.

Kanaski, J., Aksenova, M., Stoyanova, A. y Butterfield D.A. 2002.
Ferulic acid antioxidant protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems in vitro: Structure activity studies. The Journal of Nutritional Biochemistry. 13: 273–281.

Kroon, P.A. y Williamson, G. 1999. Hidroxycinnamates in plants and food: current and future perspective. Journal of Food Science and Agriculture. 79: 355-361.

Kumar-Maurya, D. y Asir-Devasagayam, T.P. 2010. Antioxidant and prooxidant nature of hydroxycinnamic acid derivatives ferulic and caffeic acids. Food and Chemical Toxicology. 48: 3369–3373.

Kumar, N., Anmad, K.K.M., Dang, R. y Husain, A. 2008. Antioxi- dant and antimicrobial activity of propolis from Tamil Nadu zone. Journal of Medicinal Plants Research. 2(12): 361-364.

Lehninger, A.L., Nelson, D.L. y Michael. M.C. 1995. Principios de Bioquímica. Segunda edición. Ediciones Omega, S.A. Barce- lona, España. pp. 1-1013.

Li-Chang, L., Yue-Wen, C. y Cheng-Chun, C. 2003. Antibacterial and DPPH free radical-scavenging activities of the ethanol extract of propolis collected in Taiwan. Journal of Food and Drug Analysis. 11(4): 277-282.

Loliger, J. 1991. The use of antioxidants in foods. In: free radicals and food additives. Aruoma, O.I. y Halliwell, B., Eds. Taylor and Francis, London, UK. pp. 121– 150.

Lou, Z., Wang, H., Rao, S., Sun, J., Ma C, y Li J. 2012. p-coumaric acid kills bacteria through dual damage mechanisms. Food Control. 25: 550-554.

Martínez-Flórez, S., González-Gallego, J., Culebras, J.M. y Tuñón, M.J. 2002. Los flavonoides: propiedades y acciones antioxi- dantes. Revisión. Nutrición Hospitalaria. 8(6): 271-278.

Mendoza-Wilson, A.M. y Glossman-Mitnik, D. 2004. CHIH-DFT determination of the molecular structure, infrared and ultraviolet spectra of the flavonoid quercetin. Journal of Molecular Structure (Theochem) 681:71–76.

Mirzoeva, O.K., Grishanin, R.N. y Calder, P.C. 1997. Antimicrobial action of propolis and some of its components: the effects on growth, membrane potential and motility of bacteria. Microbiology Research. 152: 239–46.

Moraes, C.S., Daugsch, A., Li, H., Rhim, J.S. y Park, Y.K. 2010. Com- parative antiproliferation of human prostate cancer cells by ethanolic extracts of two groups of Brazilian propolis. Ciencia y Tecnología Alimentaria. Campinas. 30(2): 539-543.

Mori, A., Nishio, C., Enoki, N., y Tawata, S. 1987.
Antibacterial activity and mode of actin of plant flavonoids against *Pro-teus vulgaris* and *Staphylococcus aureus*. Phytochemistry. 26: 2231-2234.

Ohemeng, K.A., Schwender, C.F., Fu, K.P. y Barrett, J.F. 1993. DNA gyrase inhibitory and antibacterial activity of some flavones (1). Bioorganic and Medicinal Chemistry Letters. 3:225–30.

Orsi, R.O., Sforcin, J.M., Funari, S.R., Junior A.F. y Bankova, V. 2006. Synergistic effect of propolis and antibiotics on the S. typhi. Brazilian Journal of Microbiology. 37: 108-112.

Osawa, K., Yasuda, H., Maruyama, T., Morita, H., Takeya, K., y Itokawa, H. 1992. Isoflavanones from the heartwood of *Swartzia polyphylla* and their antibacterial activity against cariogenic bacteria. Chem Pharm Bull (Tokyo). 40: 2970–2974.

Peña, R.C. 2008. Literature review: Propolis standardization: a chemical and biological review. Ciencia e Investigación Agraria. 35(1): 11-20.

Pepeljnjak, S. y Kosalec, I. 2004. Galangin expresses bactericidal activity against multiple-resistant bacteria: MRSA, *Entero- coccus* spp. and *Pseudomonas aeruginosa*. FEMS Microbiol- ogy Letters. 240: 111–116.

Sagdic, O., Silici, S. y Yetim, H. 2007. Fate of *Escherichia coli* and *E. coli* O157:H7 in apple juice treated with propolis extract. Annals of Microbiology. 57(3): 345-348.

Sato, M., Fujiwara, E. y Tsuchiya. 1996. Flavones with antibacteri- al activity against cariogenic bacteria. Journal of Ethnophar- macology. 54: 171-176.

Sato, M., Tsuchiya, H., Akagiri, M., Takagi, N. y Iinuma, M. 1997.
Growth inhibition of oral bacteria related to denture sto- matitis by anti-candidal chalcones. Australian Dental J. 42: 343-346.

Shaidi, F. y Chandrasekara, A. 2010. Hydroxycinnamates and their in vitro and in vivo antioxidant activities. Phytochemis- try Reviews. 9:147–170.

Simúth, J., Trnovský, J. y Jeloková, J. 1986. Inhibition of bac- terial DNA-dependent RNA polymerases and restriction endonuclease by UV-absorbing components from propolis. Pharmazie. 41(2), 131-132.

Takaisi-Kikuni, N.B. y Schilcher, H. 1994. Electron microscopic and microcalorimetric investigations of the possible mechanism of the antibacterial action of a defined propolis provenance. Planta Medica. 60: 222-227.

Tosi, B., Donini, A., Romagnoli, C. y Bruni, A. 1996. Antimicrobial activity of some commercial extracts of propolis prepared with different solvents. Phytotherapy Research. 10: 335-336.

Tsuchiya, H., Sato, M. y Miyazaki, T. 1996. Comparative study on the antibacterial activity of phytochemical flavanones against methicillin-resistant *Staphylococcus aureus*. Journal of Ethnopharmacology. 50:27-34.

Velázquez, C., Navarro, M., Acosta, A., Angulo, A., Dominguez, Z., Robles, R., Robles-Zepeda, R., Lugo, E., Goycoolea, F.M., Velazquez, E.F., Astiazaran, H. y Hernández, J. 2007. Anti- bacterial and free-radical scavenging activities of Sonoran propolis. Journal of Applied Microbiology. 103: 1747–1756.

Yao, L.H., Jiang, Y.M., Shi, J., Tomás-Barreran, F.A., Datta, N., Sunganusong, R. y Chen, S.S. 2004. Flavonoids in food and their health benefits. Plant Foods for Human Nutrition. 59: 113–122.

CAPÍTULO VII

Study of the molecular structural and chemical reactivity of pinocembrin by DFT calculations.

Vargas-Sánchez RD, Mendoza-Wilson AM, Balandrán-Quintana RR, Torrescano-Urrutia GR, & Sánchez-Escalante A

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Study of the molecular structure and chemical reactivity of pinocembrin by DFT calculations

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Highlights

- Structure and chemical reactivity of pinocembrin through DFT methods were studied.
- M05-2X/6-31G(d,p) afford the best quality to simulate pinocembrin structure.
- $C_{(4)}$, $O_{(4)}$ and $C_{(8)}$ were sites for nucleophilic, electrophilic and radical attack.
- Pinocembrin is capable to donate electrons, act as potential antioxidant.

ABSTRACT

Pinocembrin flavonoid ($C_{15}H_{12}O_4$), 5,7-Dihidroxyflavanone, is one of the major chemical constituents of propolis extracts which is characterized by possessing antioxidant activity. However, the relationship between its structure and antioxidant properties are little known. The principal objective of this work was to study the molecular structure and chemical reactivity (electronic affinity, *A*; ionization potential, *I*; electronegativity, χ; hardness, η; electrophilicity, ω; Fukui indices) of pinocembrin employing the density functional theory (DFT) method and different model chemistries, which include the PBEPBE, PBE1PBE, MPW1PW91, B3LYP and M05-2X functionals in combination with the 6-31G(d,p) and 6-31+G(d,p) basis set, in search of the most accurate results. This study indicate that different model chemistries were able to reproduce the molecular structure of pinocembrin compared with experimental reference data (R>0.99). It is remarkable that M05-2X/6-31G(d,p) afford the best quality to simulate pinocembrin structure. Low values of chemical potential, obtained by different model chemistries, indicate that pinocembrin is capable to donate electrons and participle as an antioxidant compound, while the local reactivity analyzed through the Fukui indices showed that $C_{(4)}$ was the preferred sites for nucleophilic attack, and $O_{(4)}$ and $C_{(8)}$ sites participle in electrophilic and radical attack.

Keywords

Pinocembrin, DFT methods, molecular structure, chemical reactivity.

1. Introduction

Flavonoids are member of a class of natural compounds that has been subject of considerable scientific interest. Many studies have suggested that flavonoids exhibit biological activities, including antibacterial, antivirial, anti-inflammatory, anticarcinogenic and antioxidant activities. However, most interest has been devoted to their antioxidant activity [1,2]. The pinocembrin molecule ($C_{15}H_{12}O_4$)

is a flavonoid known as 5,7-Dihidroxyflavanone, in nature is in its *S*-configuration. Previous studies demonstrated that pinocembrin is one of the major chemical constituents of Sonora propolis (>20 %) and the antioxidant activity of this bee product is correlated with the presence of this compound, among others [3,4].

Flavonoids, such as pinocembrin, are characterized by possesses good ability to inhibit lipid peroxidation, associating this activity to certain structural features such as the presence of phenolic groups in the A ring of the molecule and the presence of the 4-keto group in ring C [5,6]. The therapeutic effect of pinocembrin on oxidative stress in the brain of rats [7,8] finding that treatment with pinocembrina reduced the compensatory increase of superoxide dismutase (SOD) and decreased levels of malondialdehyde (MDA). Numerous reports showed the crystal structure [9], and antioxidant activity of pinocembrin (*in vitro* and biological systems), which is associated with structural characteristics of the molecule.

Many computational investigations have been made citing the successes of different Density Functional Theory (DFT) methods compared to experimental methods, in computing the molecular structure and the intrinsic reactivity of phenolic compounds, which are very important in order to disclose the relationship between the structure and chemical properties with their antioxidant activity [10-12]. Comparisons of the performance and accurate calculations of different DFT methods such as model chemistries including gradient-corrected correlation (PBEPBE), hybrid (PBE1PBE, MPW1PW91 and B3LYP) and metahybrid functional (M05-2X), with its respective basis set, allow obtain such information to understand the antioxidant properties of pinocembrin [13,14]. Unfortunately, till now, no attempt has been made to analyze the application model chemistries for accurate calculations of structure and chemical reactivity of pinocembrin, which may allow to obtain the lack of information on the intrinsic reactivity of this molecule.

Therefore, in this study, the objective of this work is to (i) perform a detailed calculation of the molecular structure and chemical reactivity of pinocembrin

flavonoid and (ii) assess the different DFT model chemistries to determine which provides satisfactory results of structural and chemical reactivity.

2. Theory and computational details

Molecular structure was designed with the program GAUSSVIEW 4.1. All calculations were performed employed the Density Functional Theory (DFT) method implemented in the GAUSSIAN 03W package [13]. Looking for more accurate model chemistry, we compare different functionals: grandient-corrected correlation (PBEPBE), hybrid (PBE1PBE, MPW1PW91 and B3LYP) and metahybrid functional (M05-2X) [14,15]. The 6-31G(d,p) basis set was used for the geometry and vibrational frequencies determination of the pinocembrin parent molecule and its radicals, anions and radical cations. From these calculations were derived some structural parameters such as bond distances, bond angles, dihedral angles, as well as some reactivity properties including total energy an thermochemical properties. Other structural parameters as shielding constants and chemical shifts by ^{13}C NMR, besides others reactivity properties among these chemical potential and fukui indices were calculated.

The reactivity properties (i.e. total energies in neutral and ionized molecule, electronic affinity, ionization potential, hardness, electronegativity and electrophilicity [chemical potential properties], Fukui indices, and shielding constants by ^{13}C NMR spectroscopy) were calculated with the 6-31+G(d,p) basis set. Shielding constants (δ) calculation was obtained by the method GIAO [16]. All calculations were performed in gas-phase (at 298 K) with the purpose of obtaining the intrinsic properties of pinocembrina (i.e., free of any interaction).

The chemical potential (μ) according to the density functional theory [17,18], is defined as the escaping tendency of electron from equilibrium:

$$\mu = (\partial^2 E/\partial N^2)_{v\,(r)} = -\chi \; ; \; \mu = -1/2\,(I + A) \qquad (1)$$

Were χ is the electronegativity.

Hardness has been defined as the resistance to charger transfer:

$$\eta = (\partial^2 E/\partial N^2)_{v\,(r)} \qquad (2)$$

These equations are related with the electronegativity (χ), describes the ability of a molecule to attract electrons towards itself in a covalent bond; and the global hardness, measure the resistance towards the deformation or polarization of the electron cloud of the atoms, ions or molecules under small perturbation of chemical reaction, through the following relation:

$$\chi = \frac{(I+A)}{2} \quad (3)$$

$$\eta = \frac{1}{2}(I - A) \quad (4)$$

Where:

$I = E(+) - E(0)$ (5)

$A = E(0) - E(-1)$ (6)

The electrophilicity index (ω), measures the capacity of chemical species to accept electrons, was calculated using the electronic chemical potential and the chemical hardness:

$$\omega = \mu^2/2\eta \quad (7)$$

The condensed Fukui functions were computed by taking the finite difference approximations from population analysis of atoms in molecules, depending on the direction of electron transfer, through the following formulas [19]:

$fk^+ = qk\ (N+1) - qk\ (N)$ [*for nucleophilic attack*] (8)

$fk^- = qk\ (N) - qk\ (N-1)$ [*for electrophilic attack*] (9)

$fk^0 = [qk\ (N+1) - qk\ (N-1)]/2$ [*for radical attack*] (10)

Were *qk* is the gross charge of atom *k* in the molecule.

3. Results and discussion

3.1 Structural properties

The representation of the molecular structure of pinocembrin molecule with its labeling and atomic numbering is shown in Fig. 1, which can be seen that structural conformation is not planar, with a torsional angle between ring B and C [$O_{(1)}$–$C_{(2)}$–$C_{(1)}$'–$C_{(2)}$'] is -32.0°. Bond interatomic distances (Å) computationally obtained are depicted in Fig. 2 and Table 1. The results were compared with experimental data of X-ray crystallography for pinocembrina [9], finding good correlation between computational and experimental bond distances and thus low standard deviations. The geometric parameters which analyzed in this work are of importance to have information about the conformation of the molecule. In the molecular structure of pinocembrina, the standard deviation (D.S) for the comparison of the calculated versus the experimental results are 0.0362 for the PBEPBE/6-31G(d,p), 0.0360 for the PBE1PBE/6-31G(d,p), 0.0363 for the MPW1PW91/6-31G(d,p), 0.0369 for the B3LYP/6-31G(d,p) and 0.0369 for the M05-2X/6-31G(d,p) model chemistry, in all cases for the interatomic bond distances. These results indicate that the calculated bond distances with DFT model chemistries are very similar to the crystal structure of pinocembrin. In agreement with our results [20] reported that M052-X/6-31+G(d,p) model chemistry successfully reproduces the molecular structure of flavonoids (quercetin).

The chemical shift (σ) obtained by ^{13}C NMR spectrometries for pinocembrina molecule are shown in Table 2, shows a typical example of the correlation between the spectral data of experimental ^{13}C NMR [21,22] and computational methods. Result showed that in both cases a linear trend with R values greater than 0.9900. The model chemistry M05-2X/6-31+G(d,p) presented the highest correlation (R=0.9958). These results confirm that the computational determination of ^{13}C NMR spectral data was very close to the experimental data, which indicate that M05-2X method it's a good tool for studies related to the determination of neutral structure molecules of phenolic compounds such as flavonoids [12,14]. In agree with Espinoza-Hicks et al. [23] hybrid (PBE1PBE)

and metahybrid (M05-2X) functionals can be used for calculation ^{13}C-NMR spectra of chalcones.

In DFT methods, the exchange-correlation energy is the main issue among all of the approximations, therefore, the accuracy of each model chemistry is depended directly by the approximate nature of the exchange-correlation energy functional. PBEPBE is classified to the generalized gradient-corrected correlation (GCA) which represents a significant improvement over the LSDA method (local density approximation). LSD method assumes that the exchange-correlation energy at any point in space and can given by the electron density of a homogeneous electron gas of the same density [24]. However, PBEPBE accuracy is still not enough for a correct description of many chemical aspects. PBE1PBE, MPW1PW91 and B3LYP are classified as Hybrid-DFT functional, which combines the exchange-correlation of 25, 25 and 20% of Hartree-Fock (HF, or exact) exchange, respectively. These functional are characterized by obtain accurate molecular structures, vibrational frequencies and bond energies[25,26]. The M05-2X functional can be called hybrid metageneralized gradient approximations (Hybridmeta-GGAs), due incorporate electron spin density, density gradient, kinetic energy density and HF exchange, which increase the accurate in molecular structures and its application in thermochemistry, kinetics, noncovalent interactions involving nonmetals [27]. Due results obtained, it is concluded that all model chemistry, mainly M05-2X/6-31+G(d,p), could be used to study the molecular structure of pinocembrin. Thus, we have used the different model chemistries for the calculation of the reactivity properties of the flavonoid considered in this work.

3.2 Chemical potential properties

These properties are a fundamental descriptor of the chemical reactivity of atoms and molecules [18] and are defined by different variables tightly related among them: electron affinity (*A*), ionization potential (*I*), electronegativity (χ), hardness (η), and electrophilicity (ω). The results for *A* and *I* of the pinocembrin molecule obtained through energy differences between the ionized and the

neutral state (Table 3), calculated at the geometry of the neutral molecule for all model chemistries ranged from *A*= -0.2917 to 0.1420 eV and *I*= 7.5244 to 8.5479 eV. The calculated values of electronegativity, global hardness, and global electrophilicity using the *I* and *A* for all model chemistries ranged from χ= 3.8332 to 4.1281 eV, η= 3.6912 to 4.4198 eV and ω= 1.8859 to 1.9903 eV. The electronic affinity (*A*) is defined as the energy released when an electron is added to a neutral molecule. A molecule or atom with high *A* values tends to take electrons easily [28,29]. The ionization potential (*I*) is defined as the amount of energy required to remove an electron of a molecule. Consequently, high values of *I* indicates that the system don not loss electrons easily [28,29]. The electronegativity (χ) measures the tendency to attract electrons in a chemical bond, as is defined as the negative of the chemical potential in DFT [17], while the hardness is measures the resistance to charge transfer [17,29]. Finally, the electrophilicity determines the affinity of molecules or atoms by electrons and measure the decay of binding energy due to a maximum electron flow between a donor and an acceptor [30].

The results obtained with the different model chemistry indicate that chemical potential properties for pinocembrin molecule were close, which are interpreted as having a low reduction potential or good antioxidant activity [31,32] due to the similarity with the (+)-catechin and (-)-epicatechin chemical potential [12]: *A*= -0.40, -0.49 eV; *I*= 7.20, 7.21 eV; χ= 3.40, 3.36; η= 3.80, 3.85 eV; ω= 1.52, 1.47 eV, respectively, obtained by the model chemistry CHI(medium)–DFT. Different results were obtained for naringenin molecule [33] which has high values of chemical potential (i.e. lower antioxidant activity than pinocembrin), this results were obtained with the M06, M06L, M06-2X and M06-HF density functional. Our results are in agreement with Hassanzadeh et al. [34] who reported that low values of chemical properties descriptors in phenolic compounds have a tendency to release the electron instead of capturing it. This tendency is a great sing for the antioxidant activity of the compounds [35].

The structure-antioxidant activity of flavonoids (flavones, flavonols, isoflavones, flavanonols and flavanones) have become an important area of study and commonly is associated with the presence of 3-hydroxyl group and 2,3-double bond in the heterocyclic ring, hydorxyl groups in rings A and B and a catechol group in ring B [35,36]. Thus, due to the lack of conjugation of flavanones provided by the 2,3-double bond with the 4-oxo group, are weak antioxidants [35]. However, pinocembrin flavanone is characterized by have in its structure a benzyl carbon [$C_{(2)}$–H or C–H] in the ring C and some investigations report that such group increases the electronegative activity of phenolic compounds, due to the presence of bridge type intramolecular interactions hydrogen, widely described in the literature for these substances [37,38]. In agreement with Mendoza et al. [12] (+)-catechin and (-)-epicatechin have in their structure a benzyl carbon and such flavonoids are characterized by have antioxidant potential similar to quercetin. Recently [34], it has been reported that the role of the structural C–H site (benzyl position) of non-flavonoid phenolic compound is not a suitable site for H–abstraction. In contrast with this results Trouillas et al. [39], reported that C–H site of keto form of quercetin have high capacity of H–transfer and consequently for the antioxidant capacity. Regarding the above, it can be confirmed the antioxidant potential that may have pinocembrin molecule.

3.3 Fukui indices

Fukui index can also be employed to determine the reactivity of each atom in the molecule, since they indicate the reactive regions as well as the nucleophilic (the site for nucleophilic attack would depend on the values of fk^{+}on the atoms with a positive charge density), electrophilic (the sites for electrophilic attack will be those atoms bearing a negative charge and where the Fukui function fk^{-} is a maximum) and radical attack (fk^{0}) in the molecule [19,40]. The analysis of Fukui indices along with the global hardness, provides a complete scheme of the reactivity of a molecule [41]. This kind of analysis was performed for each one atoms that conform pinocembrin (Table 4 and Figure 3). According to Fukui

indices all model chemistry [PBEPBE/6-31+G(d,p), PBE1PBE/6-31+G(d,p), MPW1PW91/6-31+G(d,p), B3LYP/6-31+G(d,p) and M05-2X/6-31+G(d,p)] suggest that preferred site for nucleophilic attack in pinocembrin molecule was the $C_{(4)}$ belonging to the keto group of the ring C. Carbons sites are sites adapted for nucleophilic attack, due to exists a great charge density and because carbon sites are united to an oxygen atom and this is the reason why they induce the carbocation formation, which disperses the charge by means of resonance effect to obtain its stability [42]. The most susceptible sites for electrophilic and radical attack were the $O_{(4)}$ of the keto group for the different model chemistry, except to M05-2X/6-31+G(d,p), which indicates that $C_{(8)}$ site was the preferred for electrophilic and radical attack. Based on the above results of Fukui indices of selected atoms in pinocembrin molecule the nucleophilic reactive order is:

PBEPBE/6-31+G(d,p): $C_{(4)} > H_{(7)} > C_{(7)} > H_{(6)} > H_{(5)} > C_{(5)}$

PBE1PBE/6-31+G(d,p): $C_{(4)} > C_{(7)} > C_{(9)} > C_{(5)} > H_{(3a)} > H_{(8)}$

MPW1PW91/6-31+G(d,p): $C_{(4)} > C_{(7)} > C_{(9)} > C_{(5)} > H_{(3a)} > H_{(8)}$

B3LYP/6-31+G(d,p): $C_{(4)} > C_{(7)} > C_{(9)} > C_{(5)} > H_{(6)} > H_{(7)}$

M05-2X/6-31+G(d,p): $C_{(4)} > C_{(7)} > C_{(9)} > C_{(5)} > H_{(3a)} > H_{(8)}$

The electrophilic reactivity order is:

PBEPBE/6-31+G(d,p): $O_{(4)} > C_{(8)} > C_{(4')} > C_{(6)} > O_{(5)} > O_{(7)}$

PBE1PBE/6-31+G(d,p): $O_{(4)} > C_{(8)} > C_{(6)} > C_{(4')} > O_{(5)} > O_{(1)}$

MPW1PW91/6-31+G(d,p): $O_{(4)} > C_{(6)} > C_{(4')} > O_{(5)} > O_{(1)} > C_{(3')}$

B3LYP/6-31+G(d,p): $O_{(4)} > C_{(8)} > C_{(4')} > C_{(6)} > O_{(5)} > C_{(3')}$

M05-2X/6-31+G(d,p): $C_{(8)} > O_{(7)} > C_{(6)} > O_{(4)} > O_{(1)} > O_{(5)}$

The attack for free radicals is:

PBEPBE/6-31+G(d,p): $O_{(4)} > C_{(4)} > C_{(4')} > C_{(8)} > H_{(7)} > C_{(6)}$

PBE1PBE/6-31+G(d,p): $O_{(4)} > C_{(4)} > C_{(8)} > C_{(7)} > C_{(6)} > O_{(7)}$

MPW1PW91/6-31+G(d,p): $O_{(4)} > C_{(4)} > C_{(8)} > C_{(7)} > C_{(4')} > C_{(6)}$

B3LYP/6-31+G(d,p): $O_{(4)} > C_{(4)} > C_{(8)} > C_{(7)} > C_{(4')} > C_{(6)}$

M05-2X/6-31+G(d,p): $C_{(8)} > O_{(4)} > C_{(4)} > C_{(7)} > C_{(5)} > O_{(5)}$

Differences in results of Fukui indices obtained by all chemistry model may be due to the way to calculated the exchange-correlation energy, which is the main issue among all of the approximations [15]. In agreement with our results on flavanone naringenin [33], data reported are similar to ours regarding that $C_{(4)}$ is the site preferred for nucleophilic attack and $O_{(4)}$ site play an important role in the electrophilic attack of this molecule. In other investigation rutin molecule (flavonol), Payán-Gómez et al [11] reported that preferred sites for nucleophilic attack are the $C_{(4)}$ and $C_{(2)}$, while for electrophilic attack are the $C_{(3)}$ and $C_{(4')}$ and finally for radical attack the site $C_{(3).}$ A previous computational data obtained by Mendoza-Wilson and Glossman-Mitnik [42] indicate that preferred sites for nucleophilic attack in quercetin (flavonol molecule) are the $C_{(2)}$ and $C_{(8)}$, while for electrophilic attack the $C_{(8)}$ and for radical attack the $C_{(2)}$ and $O_{(4)}$, corresponding to the oxygen of the keto group and to the carbon bond with the heteratom of oxygen in ring C (carbocation). Finally, experimental data obtained by [43] suggested that electronegative region of phenolic compounds was mainly favored in the $C_{(4)}$ of the C-ring and the 7-position of A-ring.

Electrophilic attack can occurs in aromatic carbon atoms or CH bonds [44,45], such as $C_{(8)}$ indicated in this work, and this attack can be increases with the presence of electron withdrawing groups and bond polarity. It is well known that $O_{(4)}$ group is characterized by have unparied electrons, which can lead to radical attack [46]. The free radical reactions can be influenced by polar forces

in the transition state [45]. Regarding the above, it can be confirmed the antioxidant potential that may have pinocembrin molecule.

4. Conclusions

The results presented in this investigation demonstrated that M05-2X/6-31G(d,p) and M05-2X/6-31+G(d,p) were the model chemistry that allowed more accurately calculate the structural molecule of pinocembrin such as interatomic bond distances (Å) and ^{13}C RMN chemical shifts of the molecule calculated by computational methods, due showed values very close to the experimental data of reference. The sites of interaction of pinocembrin molecule can be predicted using DFT methods trough reactivity descriptors such as the electronic affinity, ionization potential, electronegativity, hardness and electrophilicity, as well as Fukui indices, which were used to obtain a successfully description of the preferred reactive sites of pinocembrin molecule. This descriptors indicate that pinocembrin molecule easily donate electrons to free radicals, which is essential in its biological properties and antioxidant performance. In general, the MO5-2X functional is capable of reproducing intrinsic characteristics of flavonoids.

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References

1. B.H. Havsteen, The biochemistry and medical significance of the flavonoids, Pharm. Therap. 96 (2002) 67–202.

2. A. Seyoum, K. Asres, F.K. El-Fiky, Structure-radical scavenging activity relationships of flavonoids, Phytochem. 67 (2006) 2058–2070.
3. J. Hernández, F.M. Goycoolea, J. Quintero, A. Acosta, M. Castañeda, Z. Domínguez, R. Robles, L. Vázquez-Moreno, E.F. Velázquez, H. Astiazaran, E. Lugo, C. Velázquez, Sonoran propolis: Chemical composition and antiproliferative activity on cancer cell lines, Planta Med. 73 (2007) 1469–1474.
4. D. Valencia, E. Alday, R. Robles-Zepeda, A. Garibay-Escobar, J.C. Gálvez-Ruiz, M. Salas-Reyes, M. Jiménez-Estrada, E. Velázquez-Contreras, J. Hernández, C. Velázquez, Seasonal effect on chemical composition and biological activities of Sonoran propolis, Food Chem. 131 (2012) 645–651.
5. M. Neacsu, P.C. Eklund, R.E. Sjöholm, S.P. Pietarinen, M.O. Ahotupa, B.R. Holmbom, Antioxidant flavonoids from knotwood of Jack pine and European aspen, Holz Als Roh- und Werkstoff. 65 (2007) 1–6.
6. J. Zhao, Y. Li, X.F. Xue, Y. Cai, The study on the parts of higher activity of the flavones contents in propolis, Apic. of China. 56 (2005) 9–10.
7. S. Li-li, C. Bain-nian, G. Mei, Z. Heng-ai, L. Yan-jin, W. Li, D. Guan-hua, The characteristics of therapeutic of pinocembrin in transient global brain ischemia/reperfusion rats, Life Sci. 88 (2011) 521–528.
8. R. Liu, M. Gao, Y. Zhi-Hong, D. Guan-Hua, Pinocembrin protects rat brain against oxidation and apoptosis induced by ischemia-reperfusion both in vivo and in vitro, Brain Res. 1216 (2008) 104–115.
9. D.U. Guanhua, L.V. Yang, W.U. Song, W. Ke, C. Ying, Y. Zhihong, B. Yuanfeng, G. Mei, Two Types of Crystalline of Pinocembrin: A And B, Their Preparation and Their Use for Manufacture of Pharmaceutical Compositions, European Patent Application. Patent 08878072.1, 2011.
10. R.A. Laskar, I. Sk, N. Roy, N.A. Begum, Antioxidant activity of Indian propolis and its chemical constituents, Food Chem. 122 (2010) 233–237.
11. S.A. Payán-Gómez, N. Flores-Holguín, A. Pérez-Hernández, M. Piñon-Miramontes, D. Glossman-Mitnik D, Computational molecular characterization of the flavonoid rutin, Chem. cent. J. 4 (2010) 1–12.

12. AM. Mendoza-Wilson, D. Glossman-Mitnik, Theoretical study of the molecular properties and chemical reactivity of (C)-catechin and (K)-epicatechin related to their antioxidant ability, J. of Mol. Struc. THEOCHEM. 761 (2006) 97–106.
13. M.J. Frisch, G.W. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J.R. Cheeseman, J.A. Montgomery, T.K.N. Vreven, K.N. Kudin, J.C. Burant, J.M. Millam, S.S. Lyengar, J. Tomasi, V. Barone, B. Menucci, M. Cossi, G. Scalmani, N. Rega, G.A. Peterson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J.E. Knox, H.P. Hratchian, J.B. Cross, C. Adamo, J. Jaramillo, R. Gomperts, R.E. Startman, O. Yazyev, A.J. Austin, R. Cammi, C. Pomelli, J.W. Ochterski, P.Y. Ayala, K. Morokuma, G.A. Voth, P. Salvador, J.J. Dannenberg, V.G. Zakrzewski, S. Dapprich, A.D. Daniels, M.C. Strain, O. Farkas, D.K. Malick, A.D. Rabuck, K. Raghavachari, J.B. Foresman, J.V. Ortiz, Q. Cui, A.G. Baboul, S. Clifford, J. Cioslowski, B.B. Stefano, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R.L. Martin, D.J. Fox, T. Keith, M.A. Al-Laham, C.Y. Peng, A. Nanayakkara, M. Challacombe, P.M.W. Gill, B. Johnson, W. Chen, M.W. Wong, C. Gonzalez, J.A. Pople, Gaussian 03 Revision C.02, Gaussian, Inc. Wallingford C, 2004.
14. Y. Zhao, D.G. Truhlar, A density functional that accounts for medium-range correlation energies in organic chemistry, Org. Lett. 8 (2006) 5753–5755.
15. Y. Yang, H. Gao, Comparison of the performance of different DFT methods in the calculations of the molecular structure and vibration spectra of serotonin (5-hydroxytryptamine, 5-HT), J. Mol. Struc. 1013 (2012) 111–118.
16. J.R. Cheeseman, G.W. Trucks, T.A. Keith, J.M. Frisch, A comparison of models for calculating nuclear magnetic resonance shielding tensors, Chem. Phys. 104 (1996) 5497–5509.
17. R.G. Parr, W. Yang, Density Functional Theory of Atoms and Molecules. New York: Oxford University Press, 1989.
18. P. Geerlings, F. De Proft, W. Langenaeker, Conceptual density functional theory, Chem. Rev. 103 (2003) 1793–1873.

19. W. Yang, W.J. Mortier, The use of global and local molecular parameters for the analysis of the gas-phase basicity of amines, J. Am. Chem. Soc. 108 (1986) 5708–5711.
20. Z.S. Marković, J.M.D. Marković, C.B. Doličanin, Mechanistic pathways for the reaction of quercetin with hydroperoxy radical, Theor. Chem. Acc. 127(2010) 69–80.
21. J.B. Harborne, T.J. Mabry. The flavonoids: Advances in research, Hapman and Hall Ltd, London, 1982.
22. D. Bertelli, G. Papotti, L. Bortolotti, G.L. Marcazzan, M. Plessi, ^{1}H-NMR simultaneous identification of health-relevant compounds in propolis extracts, Phytochem. Anal. 23 (2011) 260–266.
23. J.C. Espinoza-Hicks, A.A. Camacho-Dávila, N.R. Flores-Holguín, G.V. Nevárez-Moorillón, D. Glossman-Mitnik, L.M. Rodríguez-Valdez, Experimental and quantum chemical studies of a novel synthetic prenylated chalcone, Chem. Cent. J. 7(2013) 17.
24. S. F. Sousa, P.A. Fernandes, M.J. Ramos, General performance of density functionals, The J. Phys. Chem. A. 111(2007) 10439–10452.
25. K.N. Kudin, G.E. Scuseria, R.L. Martin, Hybrid Density-Functional Theory and the Insulating Gap of U O 2, Phys. Rev. Lett. 89(2002) 266402.
26. B.J. Lynch, D.G. Truhlar, How well can hybrid density functional methods predict transition state geometries and barrier heights?, The J. Phys. Chem. A. 105(2001) 2936–2941.
27. Y. Zhao, N.E. Schultz, D.G Truhlar, Design of density functionals by combining the method of constraint satisfaction with parametrization for thermochemistry, thermochemical kinetics, and noncovalent interactions, J. Chem. Theor. Computation. 2(2006) 364–382.
28. J.B. Foresman, Æ. Frisch, Exploring Chemistry with Electronic Structure Methods, second ed., Gaussian, Pittsburgh, PA, 1996.
29. Raj, G. *Advanced Inorganic Chemistry Vol-1*. Krishna Prakashan Media, India, 2008.

30. R.G. Parr, L.V. Szentpály, S. Liu, Electrophilicity index, J. Ame. Chem. Soc. 121(1999) 1922–1924.
31. K. Kondo, M. Kurihara, N. Miyata, T. Suzuki, M. Toyoda, Mechanistic studies of catechins as antioxidants against radical oxidation, Arch. Biochem. Biophys. 362 (1999) 79–86.
32. R.G. Pearson, Chemical hardness and density functional theory, J. Chem. Sci. 177 (2005) 369–377.
33. D. Glossman-Mitnik, A comparison of the chemical reactivity of naringenin calculated with the M06 family of density functionals, Chem. Cent. J. 7(2013) 155.
34. K. Hassanzadeh, K. Akhtari, H. Hassanzadeh, S.A. Zarei, N. Fakhraei, K. Hassanzadeh, The role of structural CH compared with phenolic OH sites on the antioxidant activity of oleuropein and its derivatives as a great non-flavonoid family of the olive components: A DFT study, Food Chem. 164 (2014) 251–258.
35. P.G. Pietta, Flavonoids as antioxidants, J. of Nat. Prod. 63(2000) 1035–1042.
36. Z.Y. Chen, P.T. Chan, K.Y. Ho, K.P. Fung, J. Wang, Antioxidant activity of natural flavonoids is governed by number and location of their aromatic hydroxyl groups, Chem. and Phys. of Lipids, 79(1996) 157–163.
37. A. Fedorowicz, J. Mavri, P. Bala, A. Koll, Molecular dynamics study of the tautomeric equilibrium in the Mannich base, Chem. Phys. Lett. 289 (1998) 457–462.
38. J. Fals Guerra, Reactividad química de 1,3-bis-(2'-hidroxi-5'-clorobencil) imidazolidina (BISBIA) frente a fenoles y naftoles en condiciones libre de disolvente (Solvent–Free) (Doctoral dissertation, Universidad Nacional de Colombia). 2012.
39. P. Trouillas, P. Marsal, D. Siri, R. Lazzaroni, J.L. Duroux, A DFT study of the reactivity of OH groups in quercetin and taxifolin antioxidants: The specificity of the 3-OH site, Food Chem. 97(2006) 679–688.

40. D. Young, Computational Chemistry: A Practical Guide for Applying Techniques to Real World Problems, Wiley, New York, 2001.
41. J. Cruz, L.M.R. Martínez-Aguilera, R. Salcedo, M. Castro, Reactivity properties of derivatives of 2-imidazoline: an ab initio DFT study, Int. J. Quantum Chem. 85(2001) 546–556.
42. A.M. Mendoza-Wilson, D. Glossman-Mitnik, CHIH-DFT study of the electronic properties and chemical reactivity of quercetin, J. Mol. Struc.: THEOCHEM. 716(2005) 67–72.
43. J. Hyun, Y. Woo, D.S. Hwang, G. Jo, S. Eom, Y. Lee, J.C. Park, Y. Lim, Relationships between structures of hydroxyflavones and their antioxidative effects, Bio. Med. Chem. Lett. 20(2010) 5510–5513.
44. A. Viswas, K. Tewari, A Textbook Of Organic Chemistry, 3E. Vikas Publishing House Pvt Ltd, 2009.
45. R.H. Crabtree, Energy production and storage: inorganic chemical strategies for a warming world. John Wiley & Sons, 2010.
46. E.T. Denisov, T.G. Denisova, T.S. Pokidova, Handbook of free radical initiators. John Wiley & Sons, 2005.

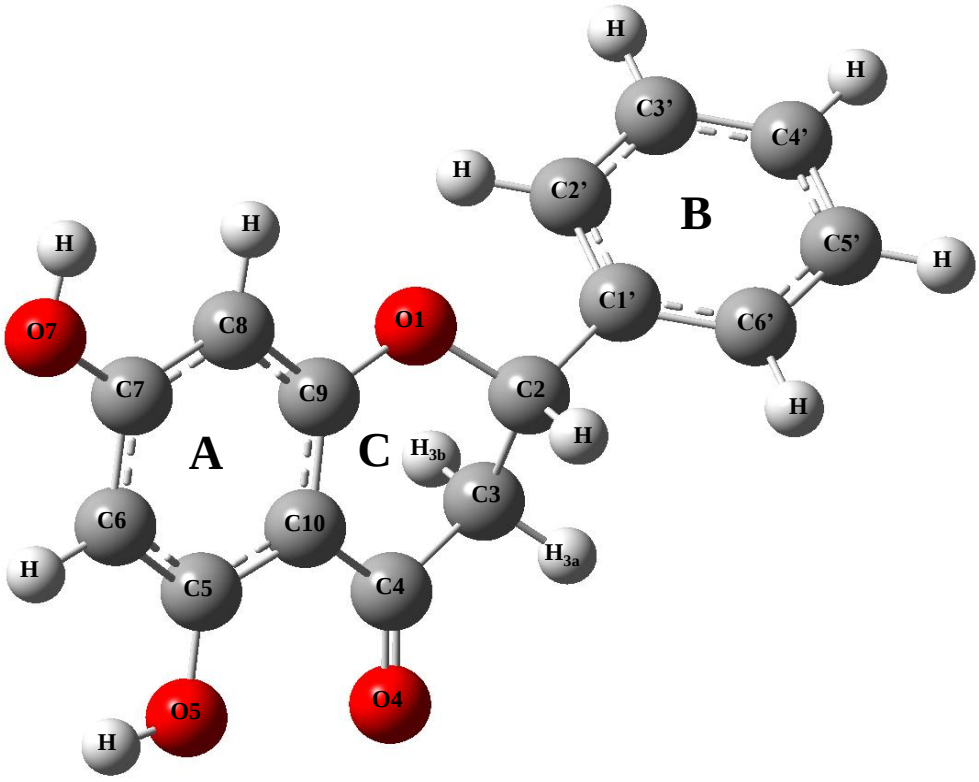

Fig. 1. Optimized molecular structure of the pinocembrin molecule.

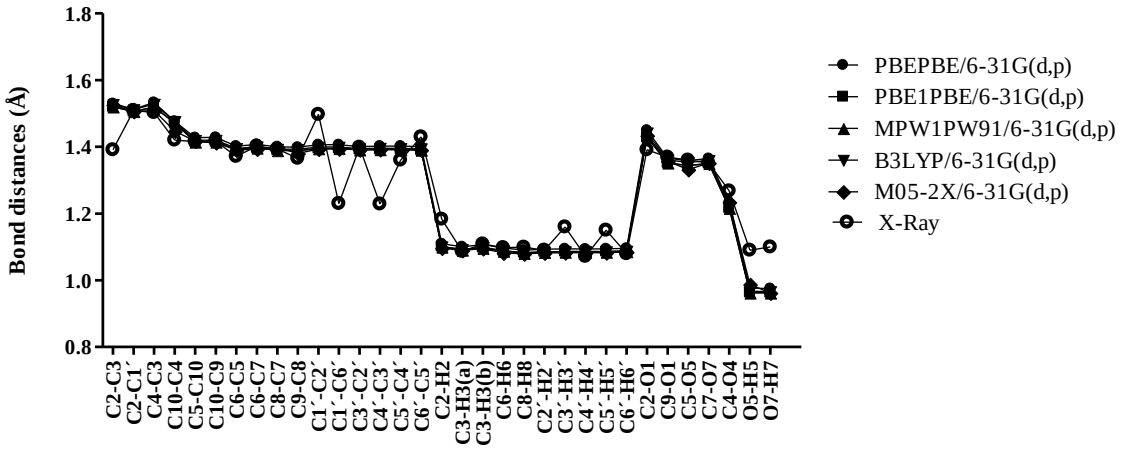

Fig. 2. Interatomic bond distances (Å) for pinocembrin molecule.

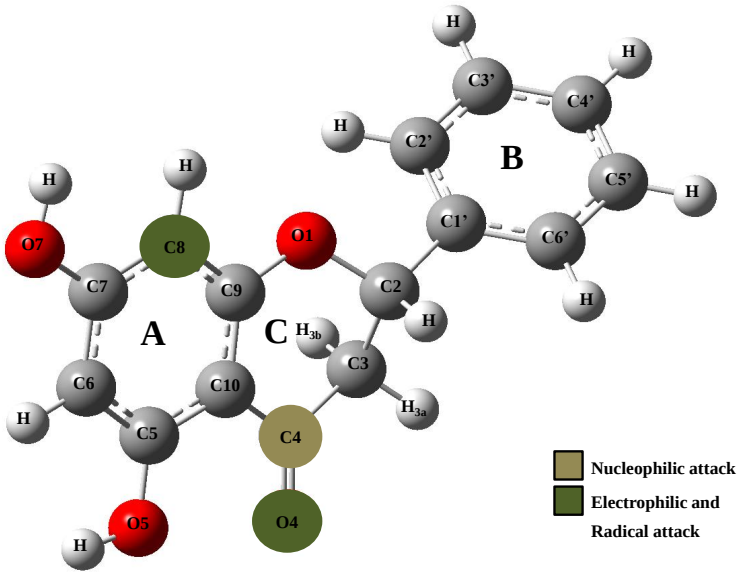

Fig. 3. Preferred sites for nucleophilic, electrophilic and radical attack in pinocembrin molecules.

Table 1
Interatomic bond distances (Å) for pinocembrin molecule.

Bond	PBEPBE/ 6-31G(d,p)	PBE1PBE/ 6-31G(d,p)	MPW1PW91/ 6-31G(d,p)	B3LYP/ 6-31G(d,p)	M05-2X/ 6-31G(d,p)	Exp. [a]
C2-C3	1.529	1.520	1.520	1.528	1.521	1.391
C3-C4	1.533	1.519	1.520	1.528	1.519	1.503
C5-C6	1.402	1.392	1.392	1.396	1.391	1.371
C6-C7	1.407	1.397	1.397	1.400	1.395	1.402
C7-C8	1.400	1.390	1.389	1.393	1.388	1.393
C8-C9	1.399	1.389	1.388	1.392	1.388	1.366
C4-C10	1.476	1.472	1.472	1.477	1.478	1.420
C5-C10	1.428	1.415	1.415	1.420	1.411	1.416
C9-C10	1.428	1.415	1.415	1.420	1.412	1.417
C2-C1´	1.513	1.506	1.506	1.512	1.506	1.509
C1´-C2´	1.406	1.395	1.395	1.400	1.394	1.497
C1´-C6´	1.406	1.396	1.395	1.399	1.394	1.230
C2´-C3´	1.401	1.392	1.391	1.395	1.392	1.399
C3´-C4´	1.402	1.392	1.392	1.396	1.392	1.229
C4´-C5´	1.402	1.392	1.392	1.396	1.392	1.360
C5´-C6´	1.401	1.391	1.391	1.395	1.391	1.430
C2-H2	1.110	1.101	1.100	1.100	1.097	1.183
C3-H3(a)	1.101	1.093	1.091	1.093	1.089	1.087
C3-H3(b)	1.107	1.098	1.097	1.098	1.095	1.109
C6-H6	1.097	1.089	1.087	1.089	1.085	1.098
C8-H8	1.091	1.083	1.081	1.082	1.079	1.099
C2´-H2´	1.093	1.085	1.084	1.085	1.082	1.090
C3´-H3´	1.094	1.086	1.085	1.086	1.083	1.160
C4´-H4´	1.094	1.086	1.085	1.086	1.083	1.072
C5´-H5´	1.094	1.086	1.085	1.086	1.083	1.150
C6´-H6´	1.096	1.088	1.086	1.087	1.085	1.080
C2-O1	1.450	1.428	1.429	1.442	1.432	1.391
C4-O4	1.232	1.217	1.216	1.221	1.213	1.268
C5-O5	1.359	1.343	1.344	1.353	1.347	1.360
C7-O7	1.365	1.350	1.350	1.360	1.354	1.351
C9-O1	1.366	1.352	1.352	1.361	1.357	1.369
O5-H5	0.977	0.963	0.963	0.967	0.962	1.090
O7-H7	0.975	0.963	0.962	0.966	0.962	1.100
DS	0.036	0.036	0.036	0.037	0.037	

[a] Crystallographic results [6].

Table 2
Shielding constant of pinocembrin molecule calculated by ^{13}C NMR spectrometries.

Carbon	Shielding constant (δ) (ppm) PBEPBE/ 6-31+G(d, p)	PBE1PBE/ 6-31+G(d, p)	MPW1PW91/ 6-31+G(d, p)	B3LYP/ 6-31+G(d, p)	MO5-2X/ 6-31+G(d, p)	Exp. [b]
C2	86.3018	80.8551	80.9118	84.9119	77.7436	78.21
C3	51.52	50.133	49.8829	51.9802	45.1153	42.77
C4	178.0953	181.1435	181.1464	182.6603	185.8566	196.45
C5	153.1887	155.7892	155.695	157.2425	158.227	164.1
C6	90.8395	91.1458	90.9441	92.4592	89.1081	96.5
C7	153.538	156.5337	156.495	158.0619	156.4097	167.23
C8	92.947	94.0478	93.9733	95.0774	88.703	95.62
C9	158.5774	161.1086	161.1927	162.943	155.7973	163.65
C10	102.9723	103.1359	103.2347	105.192	97.8126	102.3
C1´	137.5401	139.6773	139.5139	140.2649	135.3679	139.64
C2´	119.8904	122.1399	122.0037	122.9896	118.4105	127.12
C3´	123.3294	125.9666	125.6165	125.6553	122.6654	129.13
C4´	121.616	123.8881	123.6043	124.3696	120.4531	
C5´	122.1731	124.3983	124.0909	124.8593	121.1808	129.13
C6´	119.613	122.2321	121.9704	122.4462	118.1587	127.12
R	0.9893	0.9923	0.9922	0.9912	0.9958	

[b] ^{13}C NMR spectra analysis [22].

Table 3
Properties of the energy for pinocembrin molecule.

Model chemistry	Property (eV)				
	A	***I***	**χ**	**η**	**ω**
PBEPBE/6-31+G(d,p)	0.142	7.5244	3.8332	3.6912	1.9903
PBE1PBE/6-31+G(d,p)	-0.1907	8.098	3.9536	4.1444	1.8859
MPW1PW91/6-31+G(d,p)	-0.1684	8.1235	3.9775	4.146	1.908
B3LYP/6-31+G(d,p)	-0.0916	8.0158	3.9621	4.0537	1.9363
M05-2X/6-31+G(d,p)	-0.2917	8.5479	4.1281	4.4198	1.9278

A= Electronic affinity, *I*= Ionization potential, χ= Electronegativity, η= Hardness ω= Electrophilicity.

Table 4
Principal chemical reactivity sites of pinocembrin molecule obtained by using Fukui indices.

Model chemistry	Atom	Nucleophilic attack	Atom	Electrophilic attack	Atom	Radical attack
PBEPBE/6-31+G(d,p)	$C_{(4)}$	0.0722	$O_{(4)}$	0.1287	$O_{(4)}$	0.2239
PBE1PBE/6-31+G(d,p)	$C_{(4)}$	0.1017	$O_{(4)}$	0.0998	$O_{(4)}$	0.2259
MPW1PW91/6-31+G(d,p)	$C_{(4)}$	0.1023	$O_{(4)}$	0.0971	$O_{(4)}$	0.2238
B3LYP/6-31+G(d,p)	$C_{(4)}$	0.0953	$O_{(4)}$	0.1055	$O_{(4)}$	0.2246
M05-2X/6-31+G(d,p)	$C_{(4)}$	0.1207	$C_{(8)}$	0.1760	$C_{(8)}$	0.2126

CAPÍTULO VIII

Antioxidant potential of phenolic compounds of Sonoran propolis: DFT approach.

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Antioxidant potential of phenolic compounds of Sonoran propolis: DFT approach.

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ABSTRACT

Phenolic compounds are the major chemical constituents of propolis extracts (PE) and are characterized by possessing antioxidant activity. In this work the Structure-antioxidant properties relationship of flavonoids (chrysin, galangin, pinocembrin and pinostrobin) and phenolic acid (caffeic acid phenethyl ester) commonly found in PE was investigated trough M05-2X functional in conjunction with the 6-31G(d,p) and 6-31+G(d,p) basis sets, considering the structural properties, and free radical inhibition mechanism: H–atom transfer (HAT), the stepwise electron–transfer–proton–transfer (SPLET) and the sequential proton loss electron transfer (SET–PT). To complement the ability of phenolic compounds to act as antioxidant the chemical potential and Fukui indices were analyzed. Thermodynamically, the HAT mechanism contributes much in the antioxidant activity of reactive group (O–H and C–H) of phenolic compounds. All compounds presents a greater tendency to give electrons than to attract them. We found different reactive sites for nucleophilic, electrophilic and radical attack in molecules, which could mark differences in their antioxidant activity.

Keywords: Propolis, phenolic compounds, Antioxidant, M05-2X, DFT.

1. Introduction

Phenolic compounds are major bioactive constituents of the resinous and pollen fraction of propolis (bee glue), materials that account for 55% of this valuable honeybee product. Flavonoids and phenolic acid derivates represent two of the major classes of phenolic compounds of PE, which are regarded as a kind of natural compounds that has been subject of considerable scientific interest (Viuda-Martos, Ruiz-Navajas, Fernández-López, Pérez-Álvarez, & Viuda-Martos, 2008; Vargas-Sánchez et al., 2014). Many studies have suggested that these compounds obtained from PE with different solvent extraction (i.e. aqueous, methanolic, ethanolic, hydro-alcoholic, acetone, chloroform, and propilenglycol extracts) exhibit health-promoting properties, including antibacterial, antifungal, antioxidant, antiviral, anti-inflammatory and anticancer activities (Sforcin, 2007; Serra-Bonvehí & Lacalle-Gutiérrez, 2011; Jug, Zovko-Končić, & Kosalec, 2014), which given them various applications in the pharmaceutical and food industries as food additives and as nutraceuticals. However, most interest has been devoted to their antioxidant activity (Seyoum et al., 2006).

Papotti, Bertelli, Bortolotti, & Plessi (2012) investigated that several factors can influence the antioxidant activity of propolis such as the harvesting method, physicochemical composition, solvent extraction and presence of flavonoids and phenolic acid derivates such as apigenin, chrysin, galangin, kaempferol, quercetin, naringenin, pinocembrin, pinostrobin, pinobanksin, pinobanksin 3-O-acetate, *p*-coumaric acid, ferulic acid, caffeic acid, caffeic acid phenethyl ester (CAPE), and caffeic acid cinnamyl ester, compounds that also have been identified in PE from Northwest of Mexico (Hernández et al., 2007; Valencia et al. 2012; Vargas-Sanchez et al., 2014). The chemical structure of flavonoids and phenolic acid identified in propolis also play an important role in the antioxidant potential of its extracts, and this Structure-antioxidant properties relationship can be determined by several key factors implicit in their structure such as: (1) the location of the hydroxyl group in the A, B or C–ring in the molecule, (2) hydroxyl substitution in ortho–position in the B–ring alone exhibited reduced antioxidant

effect, (3) the hydroxyl substitution at the ortho–position, when accompanied by additional hydroxyl group at the para–position in the B–ring, enhanced the antioxidant action, (4) hydroxylation at para–position alone in the B-ring also gave rise to a strong antioxidant activity, (5) glycosides of flavonoids do not exhibit strong antioxidant action. A double bond is required to be present between C–2 and C–3 positions in the C–ring, (7) hydroxylation at C–3 position in C-ring contributed to the radical inhibition and (8) the benzylic position C–H is a site for slightly H abstraction (Das & Pereira, 1990; Rice-Evans, Miller, & Paganga, 1996; Wolfe, & Liu, 2008; Jiye et al., 2010; Yaung-Hung et al., 2012; Hassanzadeh et al., 2014).

The literature suggested that these structural characteristics of flavonoids and phenolic acids are implicit in the ability to scavenge free radicals (Rice-Evans, Miller, & Paganga, 1996). Therefore, there is a special interest to know the differences in the ability to scavenge free radicals of phenolic compounds found in PE. Regarding to the ability to act as free radical scavenger, three pathways commonly are discussed to explain the mechanism by which act these compounds: the first pathway involves the direct H–atom transfer (HAT), the second is associated with the stepwise electron-transfer-proton-transfer (SET–PT) and the last pathway involves the sequential proton loss electron transfer (SPLET) (Mendoza-Wilson et al., 2013). A computational evaluation of such mechanism in antioxidant molecules may exhibit different behaviors with different radicals and environments (Marcović et al., 2010). Another computational study indicate that the antiradical mechanism should be measured under the same condition to be elucidated (Marcović et al., 2010; Mendoza-Wilson et al., 2013). However, to establish the relationships of the structure and understand the mechanism by which these compound operate for free radical scavenging, it is necessary explore other reactivity parameters such as chemical potential and reactivity properties. A computational study performed by Praveena, Sadasivam, Deepha, & Sivakumar (2014) indicated that molecular descriptors such as ionization potential (IP), electron affinity (EA), Hardness (η), electronegativity (χ) and electrophilic index (ω) can be used to characterize the

intrinsic antioxidant properties of phenolic compounds. Additionally, in another study Fukui indices was used to determined which functional group of the molecule may participle in the nucleophilic, electrophilic and radical attack, variables associated with the structure, reactivity and antioxidant activity of the molecules (Mendoza-Wilson, & Glossman-Mitnik, 2005). Based on the above evidence, the computational methods (DFT) seems to be a good tool to calculate the structure and reactivity properties of phenolic compounds.

Nevertheless, the antioxidant mechanisms of phenolic compounds found in PE are unclear so that the objective of this work is through the M05-2X functional in conjunction with the 6-31G(d,p) and 6-31+G(d,p) basis sets: (i) study the molecular structure of phenolic compounds found in PE, in gas–phase, and (ii) elucidate the role of each of the three possible mechanisms, chemical potential and reactivity properties to underlying antioxidant activity.

2. Methods

2.1. Computational methodology

Molecular structure of some phenolic compound commonly found in PE such as chrysin, galangin, pinocembrin, pinostrobin and CAPE, was designed with the program GAUSSVIEW 4.1. All calculations were performed employed the Density Functional Theory (DFT) method implemented in the GAUSSIAN 03W package (Frish et al., 2004). The M05-2X metahybrid exchange-correlation functional (Zhao, Schultz, & Truhlar, 2006) in conjunction with the 6-31G(d,p) and 6-31+G(d,p) basis sets were employed for calculating geometry optimization, vibrational frequencies and reactivity properties. All calculations were performed in gas–phase (at 298 K) with the purpose of obtaining the intrinsic properties of phenolic compounds.

2.2. Thermochemical properties

In order to determinate the ability phenolic compounds identified in PE to act through the HAT mechanism, we evaluated the bond dissociation enthalpy (BDE). In gas–phase the BDE is calculated as difference in enthalpy and free

energy between the molecule (ArOH) and its radical ($ArO^{\bullet}$) after hydrogen ($H^{\bullet}$) abstraction from O–H group, applying the reaction (1) and equations (2) and (3) [Ochterski, Petersson, & Wiberg, 1995]:

$$ArOH \rightarrow ArO^{-} + H^{\bullet} \quad (1)$$

$$\Delta_r H^{\circ}\ (298\ K) = \sum(E_c + H_{corr})_{\ products} - \sum(E_e + H_{corr})_{\ reactants} \quad (2)$$

$$\Delta_r H^{\circ}\ (298\ K) = \sum(E_0 + G_{corr})_{\ products} - \sum(E_0 + G_{corr})_{\ reactants} \quad (3)$$

The ability to transfer electrons by SET-PT mechanism was determined by calculating the ionization potential (IP) from the reaction (4) [Marcović et al., 2012]:

$$ArOH \rightarrow ArOH^{\bullet+} + e^{-} \quad (4)$$

Subsequently the proton dissociation energy (PDE) was computed from the reaction (5) [Marcović et al., 2012]:

$$ArOH^{\bullet+} \rightarrow ArO^{\bullet} + H^{+} \quad (5)$$

To complement this study the ability of pinocembrin to transfer electrons by SPLET mechanism was determined. First, the proton affinity (PA) of the phenoxide anion (ArO^{-}) formed after the deprotonation (H^{+}) of the phenolic compound (ArOH) was calculated from the reaction (6) [Marcović et al., 2012]:

$$ArOH \rightarrow ArO^{-} + H^{+} \quad (6)$$

After calculate (PA), the electron transfer energy (ETE) of the phenoxide anion was calculated from the reaction (7) [Marcović et al., 2012]:

$$ArO^{-} \rightarrow ArO^{\bullet} + e^{-} \quad (7)$$

Thermochemical parameters in gas–phase were reported in terms of enthalpy (*H*) and free energy (*G*).

The different variables of the chemical potential were obtained on the differences of total electronic energies when adding or removing an electron, in relation to the neutral molecule under study (energetic-vertical procedure; Cheeseman, Trucks, Keith, & Frisch, 1996; Hussain et al., 2003;).

The condensed Fukui indices were computed by taking the finite difference approximations from population analysis of atoms in molecules, depending on the direction of electron transfer, through the following formulas [Yang, & Mortier, 1986]:

$fk^{+} = qk\ (N+1) - qk\ (N)$ [*for nucleophilic attack*] (8)

$fk^{-} = qk\ (N) - qk\ (N-1)$ [*for electrophilic attack*] (9)

$fk^{0} = [qk\ (N+1) - qk\ (N-1)]/2$ [*for radical attack*] (10)

Were *qk* is the gross charge of atom *k* in the molecule.

3. Results and discussion

3.1. Molecular structural properties

Antioxidant properties relationship of flavonoids and phenolic acids was investigated. However, a knowledge of the geometries of the phenolic compound is necessary for investigate the structure-activity relationship (Vafiadis, & Bakalbassis, 2003; Modak, Leonor-Contreras, González-Nilo, & Torres, 2005; Wolfe, & Liu, 2008). The optimized molecular structure showing the atomic labeling and numbering are showed in the Fig. 1 and Fig. 2. Results for the equilibrium conformation of the neutral molecule of chrysin, galangin, pinocembrin, pinostrobin and CAPE calculated with the M05-2X/6-31G(d,p) are reported in Table 1. These results were compared with the experimental X–ray crystallography data references (Shoja, 1989; Son, Lobkowsky, & Lewis, 2001; Yamovoi, Kul'magambetova, Kulyyasov, Turdybekov, & Adekenov, 2001; Guanhua et al., 2010). The standard error of the differences between the experimental and the calculated bond lengths and bond angles was very low (<0.005 Å), which confirm good structural reproduction in all molecules evaluated.

The results also indicate that conformation of the neutral molecule of chrysin and galangin show a not planar structure, whose tensional angle between ring B and C [$O_{(1)}$–$C_{(2)}$–$C_{(1)}$'–$C_{(2)}$'] is 23.0 and -7.2°, while the flavanones pinocembrin and pinostrobin show a similar torsional angle (-32.0 and -32.3°, respectively). The structure of CAPE has two rings, A and B. Both rings show a irregular structure with a torsional angle of 0.46° [$C_{(1)}$–$C_{(6)}$–$C_{(7)}$–$C_{(8)}$] and 90.2° [$C_{(10)}$–$C_{(11)}$–$C_{(12)}$–$C_{(17)}$].Therefore, CAPE has a more twisted structure. In agreement with our results a computational study performed by Mendoza–Wilson, Castro-Arredondo, & Balandrán-Quintana (2014) demonstrate that molecular structures

of procyanidins and distintic types of subunits could be optimized through M05-2X functional in combination with the 6-31G(d,p) basis set; and the relationship of the molecular structure with the ability to inhibit free radicals can be established through that chemical model by DFT method. The M05-2X metahybrid exchange-correlation functional, incorporate electron spin density, density gradient, kinetic energy density, and Hartree-Fock (HF) exchange (Zhao, Schultz, & Truhlar, 2006). This functional is characterized by has good accurate in the calculation of the geometric structure and energies such as thermochemistry properties (Wodrich, Corminboeuf, Schreiner, Fokin, & Schleyer, 2007).

3.2. Thermochemical properties

Thermochemical parameters are important parameters applied to understanding the mechanisms of free radical inhibition in phenolic compounds (Marcović et al., 2012). Considering that the first reaction site can make a difference in antioxidant activity, depending on the mechanism followed, the ability to inhibit free radicals (HAT, SET–PT and SPLET (expressed in terms of reaction enthalpies and free energy BDE, PA and IP) of the main constituents of PE was determined. In the HAT mechanism, the relative energies of different radicals (BDE) obtained after abstraction of a hydrogen atom from each reactive group (O–H or C–H) of chrysin, galangin, pinocembrin, pinostrobin and CAPE are show in Table 2. The most stable radical for the flavonols chrysin and galangin molecules was the 7–ArO$^{\bullet}$ and 3–ArO$^{\bullet}$ (94 and 86 kcal/mol, respectively), while for the flavanones pinocembrin and pinostrobin was the same (2–ArC$^{\bullet}$, belonging to benzyl carbone; 85 and 84 kcal/mol). The presence of a methoxy group (OCH_3) in A-ring of pinostrobin molecule do not influenced in its BDE values. These results are a remarkable case in flavonoids, due the H-atom transfer from 2–CH group of C-ring is easier than 7–OH and 3–OH group from chrysin and galangin. However, the phenolic acid (CAPE) presented the most stable radical (3–ArO$^{\bullet}$, beloging to catechol group; 76 kcal/mol) than flavonoids compounds, which indicate remarkably greater O–H bond breaking.

H-atom transfer is a major mechanism by which phenolic compounds exerts its antioxidant potential, H-atom directly transfers from antioxidant to active free radical to break chain reaction (Marcović et al., 2012). In gas–phase, for molecules analyzed the BDE sequence is as follow:

Chrysin: 7–OH > 5–OH > 8–CH

Galangin: 3–OH > 7–OH > 5–OH > 8–CH

Pinocembrin: 2–CH > 5–OH > 7–OH > 8–CH

Pinostrobin: 2–CH > 5–OH > 8–CH

CAPE: 3–OH > 2–OH

It has been reported in the literature that phenolic acid (CAPE) > flavonols > flavanones can act as antioxidant molecules to inhibit peroxyl radical formation and such differences are associated to their molecular structure and each reactive group (Russo, Longo, & Vanella, 2002; Hyun et al., 2010). Dziedzic, & Hudson (1983) indicated that the antioxidant activity of phenolic acids and their esters depends on the number of hydroxyl groups in the molecule that would be strengthened by steric hindrance. Theoretical studies demonstrate that ability of phenolic compounds to inhibit free radical is widely associated to the phenolic O–H group than C–H group (Hassanzadeh et al., 2014). However, our results demonstrated the great contribution of C–H group in the H-atom transfer of flavanones molecules. Based on the values of BDE, is suggested that the most important structural factors of the phenolic compounds associated with the mechanism HAT are the presence of hydroxyl and chatecol groups, and relevantly the presence of the C–H group.

Regarding the SPLET mechanism (Table 2), the proton affinity (PA) value of the phenoxide anion (ArO^{-}) was calculated after deprotonation (H^{+}) of each reactive group (O–H or C–H). The results indicates that phenoxide anion of CAPE (3–ArO^{-}; A-ring) seems to having the lowest PA value (331 kcal/mol) and for flavonoid chrysin, galangin, pinocembrin and pinostrobin the groups 7–ArO^{-} (339 kcal/mol), 7–ArO^{-} (334 kcal/mol), 5–ArO^{-} (338 kcal/mol) and 5–ArO^{-} (346 kcal/mol), were the most important for SPLET mechanism. OCH_3 substituent in A-ring show a significantly influence on PA value of 5–ArO^{-} from pinostrobin.

In gas–phase, the PA sequence of phenolic compound is as follow:

Chrysin: 7–OH > 5–OH > 8–CH

Galangin: 7–OH > 3–OH > 5–OH > 8–CH

Pinocembrin: 7–OH > 5–OH > 2–CH > 8–CH

Pinostrobin: 5–CH > 2–CH > 8–CH

CAPE: 3–OH > 2–OH

This results indicate that the most important reactive group in SPLET mechanism are the O–H. The SPLET mechanism is an important antioxidant mechanism. The first proton loss process, which is governed by the acid strength of phenolic O–H, is the crucial step of this mechanism and the phenoxide anion (ArO^{-}) obtained, is a electron donor which facilitates the following electron transfer process (Litwinienko, & Ingold, 2007). In agree previous computational research showed that the O–H group play an important role in free radical scavenging activity of anthocyanidins (Lu, Qiang, Li, Zhang, & Zhang, 2014)

In addition to HAT and SPLET mechanisms, the SET-PT mechanisms, described by the IP value (Table 2), is have been often used for predicting the free radical inhibition (Marcović et al., 2012). Lower IP values indicate the most stable radical cation ($ArOH^{\bullet+}$). In gas–phase, the IP values for phenolic antioxidants indicated that the most stable radical cation was obtained in the following order: galangina (174 kcal/mol), chrysin (176 kcal/mol), CAPE (177 kcal/mol), pinostrobin (182 kcal/mol) and pinocembrin (183 kcal/mol). The IP of flavanones molecules higher than that flavonols and phenolic acid by approximately 10 kcal/mol. This indicates that flavanones have a weaker electron-donating capacity. According to these results, in gas–phase, the potential work mechanism of phenolic compounds found in PE was the H-atom transfer (HAT). In agree with our results a computational study performed by Mendoza-Wilson et al. (2013) indicates that, in gas–phase, the prevailing mechanism of some phenolic compounds of apple peel was the HAT, follow by SET-PT and SPLET.

3.3. Chemical potential properties

Within the conceptual framework of DFT, the commonly used chemical potential indices are the electronic affinity (*A*), ionization potential (*I*), electronegativity (χ), hardness (η) and electrophilicity (ω), which can play an important role in the understanding the chemical reactivity or antioxidant potential of phenolic compounds (Geerlings et al., 2003), due to of some of these descriptors measuring the tendency to capturing or donate electrons (Foresman, & Frisch, 1996). The chemical potential are show in the Table 3, indicating that values for all variables associated with the chemical potential are low and similar between the molecules analyzed (standard error <0.5 eV). These results indicate that phenolic compounds have a tendency to give electrons, which is associated with the antioxidant potential. In agreement with our results Payán-Gómez, Flores-Holguín, Pérez-Hernández, Piñon-Miramontes, & Glossman-Mitnik (2010) obtained a low reduction potential for rutin: *A*= 0.067 eV, *I* = 7.284 eV, χ = 3.679 eV, η= 3.158 eV and ω= 2.143 eV. In another computational study performed by Martínez-Araya (2012), Caffeic acid exhibited close values to those obtained for CAPE: η= 4.155 eV and ω= 1.690 eV.

Electron affinity (*A*) is positive, which means that the anion is more stable than the neutral molecule, that is, they are more capable of accepting electrons, and thus, they represent the most efficient antiradicals (expressed as their electron-accepting capacity). The low ionization potential (*I*) values represent the most easily oxidized substances and indicate the most efficient antiradicals, expressed in terms of their electron-donating capacity. In case of the electronegativity (χ), high values imply a strong capacity to donate electrons. For hardness descriptor (η), high values indicate strong resistance to charge transfer, while low electrophilicity values (ω), indicate a high capacity to donate electrons (Reed, 1997; Martínez, 2009).

3.4. Fukui indices

The reactivity of phenolic compounds was determined through Fukui indices, since they indicate the reactive regions of the molecule as well as the nucleophilic, electrophilic and radical attack in the molecule (Yang, & Mortier, 1986). This analysis was performed for each atom that conform the molecule and the results are show in Table 4. According to Fukui indices the preferential sites for the nucleophilic, electrophilic and radical attack are as following: chrysin [$H_{(7)}$, $O_{(7)}$, $H_{(6)}$], galangin [$H_{(4')}$, $O_{(7)}$, $H_{(5')}$], pinocembrin [$C_{(4)}$, $C_{(8)}$, $C_{(8)}$], pinostrobin [$H_{(4')}$, $O_{(1)}$, $H_{(3)}$] and CAPE [$H_{(15)}$, $O_{(9)}$, $H_{(15)}$], respectively.

Based on these results, the major preferential site for all attack type in chrysin molecule is the A-ring, and for galangin B-ring are preferential for nucleophilic and radical attack, while A-ring for electrophilic attack. In flavanones molecules, despite having similar values of thermochemical and chemical potential properties, these compounds had different reactive sites. C-ring was the preferential site for nucleophilic attack for pinocembrin, while A-ring was the preferential for electrophilic and radical attack. In pinostrobin B-ring was preferential for nucleophilic attack, while C-ring was preferential site for electrophilic and radical attack. The preferential sites for nucleophilic and radical attack in CAPE molecule was the B-ring. In agreement with our results Mendoza-Wilson, & Glossman-Mitnik (2005) indicate that the more reactive sites, that can be related to the antioxidant properties, are located in B and C-rings. In another computational study Payán-Gómez S (2010), reported that in rutin the preferential site for nucleophilic and electrophilic attack is on C-ring, while for radical attack the A-ring governed.

4. Conclusions

In this work we have reported the results of a DFT–study for the determination of antioxidant mechanism of phenolic compounds indentified commonly in propolis extracts such as flavonoids (chrysin, galangin, pinocembrin and pinostrobin) and phenolic acid (CAPE). The results obtained by the M05-2X functional in conjunction with the 6-31G(d,p) and 6-31+G(d,p) basis

sets, revealed that the functional groups (O–H and C–H) of phenolic compounds with high potential to inhibit free radicals by the dominant mechanism (HAT), but also has potential for SPLET < SET–PT mechanism, are as following: for CAPE the 3–OH, for galangin the 3–OH, for chrysin 7–OH group, for pinostrobin the 2–CH and for pinocembrin the 2–CH. Which indicates that not only the Ar–OH groups, but also Ar–CH, can participate in the antioxidant activity of the molecule. Through antioxidant potential descriptors phenolic compounds are similar, and this molecules have a tendency to give electrons. The results in Fukui indices indicate that B and C-rings are the preferential reactive sites. Computational data obtained with M05-2X/6-31G(d,p) and M05-2X/6-31G(d,p) model chemistry are considering good tools for the determination of structural properties, mechanisms antioxidants, chemical potential properties and reactive sites of phenolic compounds related to their antioxidant potential.

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References

Cheeseman, J.R., Trucks, G.W., Keith, T.A., & Frisch J.M. (1996). A comparison of models for calculating nuclear magnetic resonance shielding tensors. *Chemical Physics*,*104*, 5497–5509.

Das, N.P., & Pereira, T.A. (1990). Effects of flavonoids on thermal autoxidation of palm oil: structure-activity relationships. *Journal of American Oil Chemists Society*, *67*, 255–258.

Dziedzic, S. Z.; Hudson, B. J. F. (1983). Polyhydroxychalcones and flavanones as antioxidants for edible foods. *Food Chemistry*, *12*,205–212.

Foresman, J. B., & Frisch A.E. (1996) Exploring Chemistry with Electronic Structure Methods, second ed., Gaussian Inc., USA.

Geerlings, P., De Proft, F., & Langenaeker, W. (2003). Conceptual density functional theory. *Chemical reviews*, *103*, 1793–1874.

Guanhua, D. U., Yang, L. V., Song, W. U., Ke, W., Ying, C., Zhihong, Y., Yuanfeng B., & Mei, G.(2011). Two Types of Crystalline of Pinocembrin: A And B, Their Preparation and Their Use for Manufacture of Pharmaceutical Compositions. *European Patent Application*. Patent 08878072.1

Hassanzadeh, K., Akhtari, K., Hassanzadeh, H., Amir-Zarei, S., Fakhraei, N., Hassanzadeh, K. (2014). The role of structural C–H compared with phenolic OH sites on the antioxidant activity of oleuropein and its derivatives as a great non-flavonoid family of the olive components: A DFT study. *Food Chemistry*, *164*, 251–258.

Hernández, J., Goycoolea, F. M., Quintero, J., Acosta, A., Castañeda, M., Domínguez, Z., Robles, R, Vázquez-Moreno, L., Velázquez, E. F., Astiazaran, H., Lugo, E., & Velázquez, C. (2007). Sonoran propolis: Chemical composition and antiproliferative activity on cancer cell lines. *Planta Medica*, *73*, 1469–1474.

Hussain, H. H., Babia, G., Durst, T., Wright, J. S., Flueraru, M., Chichirau, A., & Chepelev, L. L. (2003). Development of Novel Antioxidants: Design, Synthesis, and Reactivity. Journal of Organic Chemistry, 68, 7023–7032.

Hyun, J., Woo, Y., Hwang, D. S., Jo, G., Eom, S., Lee, Y., Park, JC., & Lim, Y. (2010). Relationships between structures of hydroxyflavones and their antioxidative effects. *Bioorganic & medicinal chemistry letters*, *20*(18), 5510–5513.

Jiye, H., Yoonkyung, W., Do-seok, H., Geunhyeong, J., Sunglock, E., Younggiu, L., Jun, C. P., & Yoongho, L. (2010). Relationships between structures of hydroxyflavones and their antioxidative effects. *Bioorganic and Medicinal Chemitry Letters*, *20*, 5510–5513.

Jug, M., Zovko-Končić, M., & Kosalec, I. (2014). Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. LWT-*Food Science and Technology*, *57*, 530–537.

Litwinienko, G., & Ingold, K. U. (2007). Solvent effects on the rates and mechanisms of reaction of phenols with free radicals. *Accounts of chemical research*, *40*, 222–230.

Lu, L., Qiang, M., Li, F., Zhang, H., & Zhang, S. (2014). Theoretical investigation on the antioxidative activity of anthocyanidins: A DFT/B3LYP study. *Dyes and Pigments*, *103*, 175–82.

Marković , Z., Milenović, D., Dorović , J., Di mitrić Marković, J.M., Stepanić, V., Lučić, B., & Amić , D. (2012). PM6 and DFT study of free radical scavenging of morin. *Food Chemistry*, *134*, 1754–1760.

Martínez, A. (2009). Donator− Acceptor Map and Work Function for Linear Polyene-Conjugated Molecules. A Density Functional Approximation Study. *The Journal of Physical Chemistry B*, *113*, 3212–3217.

Martínez-Araya, J. I. (2012). Revisiting caffeate's capabilities as a complexation agent to silver cation in mining processes by means of the dual descriptor–a conceptual DFT approach. *Journal of molecular modeling*, *18*, 4299–4307.

Mendoza-Wilson, A. M., & Glossman-Mitnik, D. (2005). CHIH-DFT study of the electronic properties and chemical reactivity of quercetin. *Journal of Molecular Structure: THEOCHEM*, *716*, 67-72.

Mendoza-Wilson, A. M., Armenta-Vázquez, M. E., Castro-Arredondo, S. I., Espinosa-Plascencia, A., Robles-Burgueno, M. R., González-Rios, H., González-León, A., & Balandran-Quintana. R. R. (2013). Potential of polyphenols from an aqueous extract of apple peel as inhibitors of free radicals: An experimental and computational study. *Journal of Molecular Structure*, *1035*, 61–68.

Mendoza–Wilson, A. M., Castro-Arredondo, S. I., & Balandrán-Quintana, R. R. (2014). Computational study of the structure–free radical scavenging relationship of procyanidins. *Food chemistry*, *161*, 155–161.

Modak, B., Leonor-Contreras, M., González-Nilo, F., & Torres, R. (2005). Structure–antioxidant activity relationships of flavonoids isolated from the resinous exudate of *Heliotropium sinuatum*. *Bioorganic & medicinal chemistry letters*, *15*, 309–312.

M.J. Frisch, G.W. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J.R. Cheeseman, J.A. Montgomery, T.K.N. Vreven, K.N. Kudin, J.C. Burant, J.M. Millam, S.S. Lyengar, J. Tomasi, V. Barone, B. Menucci, M. Cossi, G. Scalmani, N. Rega, G.A. Peterson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J.E. Knox, H.P. Hratchian, J.B. Cross, C. Adamo, J. Jaramillo, R. Gomperts, R.E. Startman, O. Yazyev, A.J. Austin, R. Cammi, C. Pomelli, J.W. Ochterski, P.Y. Ayala, K. Morokuma, G.A. Voth, P. Salvador, J.J. Dannenberg, V.G. Zakrzewski, S. Dapprich, A.D. Daniels, M.C. Strain, O. Farkas, D.K. Malick, A.D. Rabuck, K. Raghavachari, J.B. Foresman, J.V. Ortiz, Q. Cui, A.G. Baboul, S. Clifford, J. Cioslowski, B.B. Stefano, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R.L. Martin, D.J. Fox, T. Keith, M.A. Al-Laham, C.Y. Peng, A. Nanayakkara, M. Challacombe, P.M.W. Gill, B. Johnson, W. Chen, M.W. Wong, C. Gonzalez, J.A. Pople, Gaussian 03 Revision C.02, Gaussian, Inc. Wallingford C, 2004.

Oschterski, J.W., Petersson, G.A. & Wiberg, K. (1995). A comparison model chemistries. *Journal of American and Chemical Society*, *117*, 11299-11308.

Papotti, G., Bertelli, D., Bortolotti, D., & Plessi, M. (2012). Chemical and functional characterization of Italian propolis obtained by different harvesting methods. *Journal of Agriculture and Food Chemistry*, *60*, 2852–2862.

Payán-Gómez, S. A., Flores-Holguín, N., Pérez-Hernández, A., Piñon-Miramontes, M., & Glossman-Mitnik, D. (2010). Computational molecular characterization of the flavonoid rutin. *Chemistry Central Journal*, *4*, 1–12.

Praveena, R., Sadasivam, K., Deepha, V., & Sivakumar, R. (2014). Antioxidant potential of orientin: A combined experimental and DFT approach. *Journal of Molecular Structure*, *1061*, 114-123.

Reed, J. L. (1997). Electronegativity: Chemical Hardness I. *The Journal of Physical Chemistry A*, *101*, 7396-7400.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free radical biology and medicine*, *20*, 933–956.

Russo, A., Longo, R., & Vanella, A. (2002). Antioxidant activity of propolis: role of caffeic acid phenethyl ester and galangin. *Fitoterapia*, *73*, S21–S29.

Serra-Bonvehí, J., & Lacalle-Gutiérrez, A. (2011). Antioxidant activity and total phenolics of propolis from the basque country (Northeastern Spain). *Journal American Oil Chemists Society*, *88*, 1387–1395.

Seyoum, A., Asres, K., & El-Fiky, F. K. (2006). Structure-radical scavenging activity relationships of flavonoids. *Phytochemistry*, *67*, 2058–2070.

Sforcin, J. M. (2007). Propolis and the immune system: a review. *Journal of Ethnopharmacology*, *113*, 1–14.

Shoja, M. (1989). 5-Hydroxy-7-Methoxyflavone. *Acta Crystallographica*, *45*,828–829

Son, S., Lobkowsky, E. B., & Lewis, B. A. (2001). Caffeic Acid Phenethyl Ester (CAPE): Synthesis and X–Ray Crstallographic Analysis. *Chemical and pharmaceutical bulletin*, *49*, 236–238.

Vafiadis, A. P., & Bakalbassis, & E. G. (2003). A computational study of the structure-activity relationships of some p-hydroxybenzoic acid antioxidants. *Journal of the American Oil Chemists Society*, *80*, 1217–1223.

Valencia, D., Alday, E., Robles-Zepeda, R., Garibay-Escobar, A., Galvez-Rui, J. C., Salas-Reyes, M., Jiménez-Estrada, M., Velázquez-Contreras, E., Hernández, J., & Velázquez, C. (2012). Seasonal effect on chemical composition and biological activities of Sonoran propolis. *Food Chemistry*, *131*, 645–651.

Vargas-Sánchez, R. D., Torrescano-Urrutia, G. R., Acedo-Félix, E., Carvajal-Millán, E., González-Córdova, A. F., Vallejo-Galland, B., Torres-Llanez, M. J., & Sánchez-Escalante, A. (2014). Antioxidant and antimicrobial activity of

commercial propolis extract in beef patties. *Journal of Food Science*, *79*, C1499–C1504.

Vargas-Sánchez, R. D., Torrescano-Urrutia, G. R., Mendoza-Wilson, A. M., Vallejo-Galland, B., Acedo-Félix, E., Sánchez-Escalante, J. J., Peñalba-Garmendia, M. C., & Sánchez-Escalante, A. (2014). Mechanisms involved in antioxidant and antibacterial activity of propolis. *Biotecnia*, *16*, 32–37.

Viuda-Martos, M., Ruiz-Navajas, Y., Fernández-López, J., & Pérez-Álvarez, J. A. (2008). Functional properties of honey, propolis, and royal jelly. *Journal of Food Science*, *73*, 117–124.

Wodrich, M. D., Corminboeuf, C., Schreiner, P. R., Fokin, A. A., & Schleyer, P. V. R. (2007). How accurate are DFT treatments of organic energies?. *Organic letters*, *9*, 1851-1854.

Wolfe, K. L., & Liu, R. H. (2008). Structure− activity relationships of flavonoids in the cellular antioxidant activity assay. *Journal of agricultural and food chemistry*, *56*, 8404–8411.

Yamovoi, V. I., Kul'magambetova, E. A., Kulyyasov, A. T., Turdybekov, K. M., & Adekenov, S. M. (2001). Molecular structure of a novel polymorphic modification of pinostrobin. *Chemistry of natural compounds*, *37*, 424–427.

Yang, W., & Mortier, W. J. (1986). The use of global and local molecular parameters for the analysis of the gas–phase basicity of amines. *Journal of American and Chemical Society*, *108*, 5708–5711.

Yaung-Hung, C., Zhi-Shiang, Y., Chi-Chung, W., Yeong-Sheng, C., Bo-Cheng, W., Chih-Ang, H., & Tzenge-Lien, S. (2012). Evaluation of the structure–activity relationship of flavonoids as antioxidants and toxicants of zebrafish larvae. *Food Chemistry*, *134*, 717–724.

Zhao, Y., Schultz, N. E., & Truhlar, D. G. (2006). Design of density functionals by combining the method of constraint satisfaction with parametrization for thermochemistry, thermochemical kinetics, and noncovalent interactions. *Journal of Chemical Theory and Computation*, *2*, 364–382.

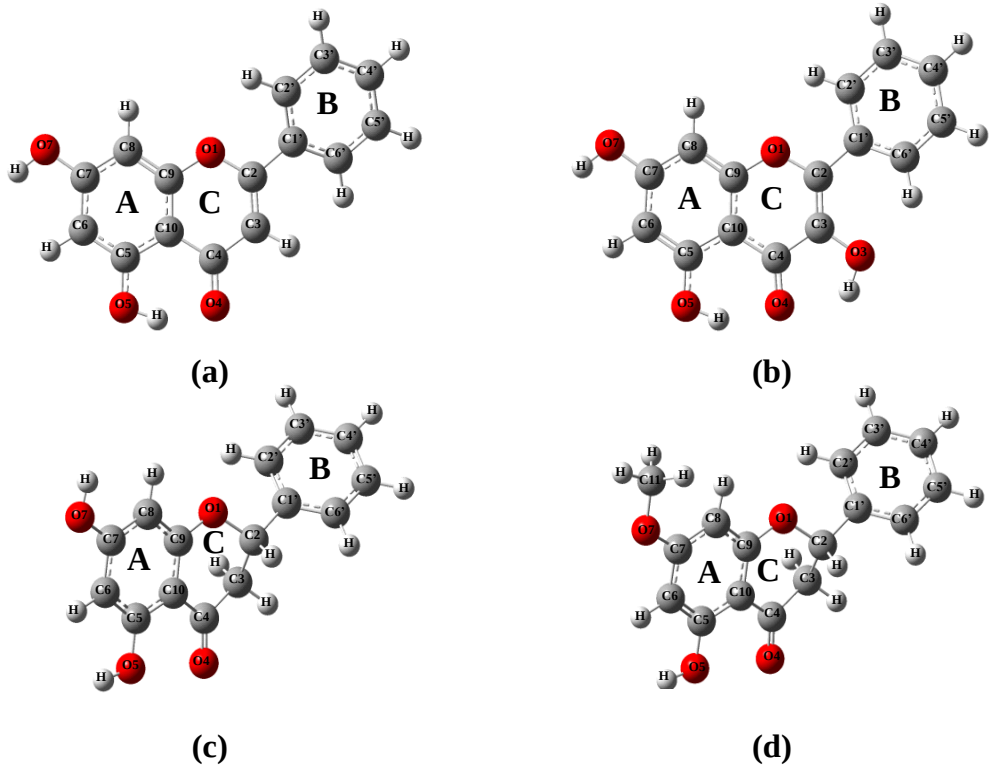

Fig. 1. Optimized molecular structure of phenolic compounds (**a**, chrysin; **b**, galangin; **c**, pinocembrin; **d**, pinostrobin) in gas–phase.

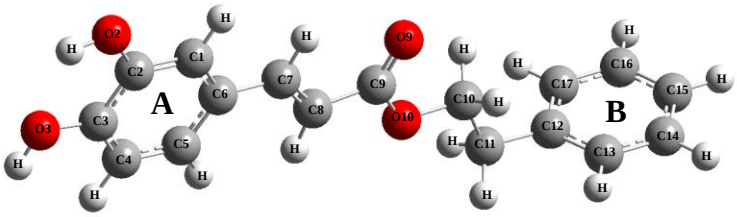

Fig. 2. Optimized molecular structure of CAPE in gas–phase.

Table 1

Interatomic bond distances of phenolic compounds (in gas-phase) indentified in propolis extracts.

Bond	Chrysin		Bond	Galangin		Bond	Pinocembrin		Bond	Pinostrobin		Bond	CAPE	
length	Theor.	Exp.[a]	length	Theor.	Exp.[b]	lenght	Theor.	Exp.[c]	length	Theor.	Exp.[d]	length	Theor.	Exp.[e]
C3-C4	1.45	1.44	C2-C4	1.45		C2-C3	1.52	1.39	C5-C6	1.39	1.41	C3-C4	1.39	
C2-C3	1.35	1.34	C3-C2	1.36		C2-C1´	1.51	1.51	C6-C7	1.40	1.38	C3-C2	1.40	
C3-H3	1.08		C3-O3	1.35		C4-C3	1.52	1.50	C6-H6	1.08		C3-O3	1.37	1.37
C4-C10	1.45	1.44	C4-C10	1.43		C10-C4	1.48	1.42	C5-C10	1.42	1.43	C4-C5	1.39	1.39
O4-C4	1.24	1.26	C4-O4	1.25		C5-C10	1.41	1.42	O5-C5	1.35	1.35	C4-H4	1.08	
C9-C10	1.40	1.38	C10-C9	1.40		C10-C9	1.41	1.42	C9-C10	1.41	1.38	C5-C6	1.40	
C5-C10	1.42	1.42	C10-C5	1.42		C6-C5	1.39	1.37	C4-C10	1.48	1.47	C5-H5	1.08	
C8-C9	1.38	1.39	C9-C8	1.39		C6-C7	1.40	1.40	C8-C9	1.39	1.41	C1-C6	1.40	1.39
O1-C9	1.37	1.39	C9-O1	1.35		C8-C7	1.39	1.39	O1-C9	1.36	1.40	C6-C7	1.46	
C1'-C2	1.47	1.49	C2-C1'	1.47		C9-C8	1.39	1.37	C7-C8	1.39	1.40	C1-C2	1.38	1.39
O1-C2	1.35	1.37	C2-O1	1.37		C1´-C2´	1.39	1.50	C8-H8	1.08		C1-H1	1.08	
C5-C6	1.39	1.36	C5-C6	1.38		C1´-C6´	1.39	1.23	O7-C7	1.35	1.41	C2-O2	1.36	1.36
O5-C5	1.33	1.35	C5-O5	1.33		C3´-C2´	1.39	1.40	C3-C4	1.52	1.53	O3-H3	0.96	
C7-C8	1.39	1.40	C8-C7	1.39		C4´-C3´	1.39	1.23	O4-C4	1.21	1.23	C7-C8	1.34	
C8-H8	1.08		C8-H8	1.08		C5´-C4´	1.39	1.36	C2-C3	1.52		C7-H7	1.09	
C6-C7	1.40	1.39	C7-C6	1.40		C6´-C5´	1.39	1.43	C2-C1'	1.51	1.51	C8-H8	1.08	
O7-C7	1.35	1.37	C7-O7	1.35		C2-H2	1.10	1.18	O1-C2	1.43	1.44	C8-C9	1.48	
C6-H6	1.08		C6-H6	1.08		C3-H3(A)	1.09	1.09	C2-H2	1.10		C9-O9	1.21	1.22
C5'-C6'	1.39	1.39	C5'-C6'	1.39		C3-H3(B)	1.10	1.11	C3-H3(A)	1.09		C9-O10	1.35	1.32
C4'-C5'	1.39	1.37	C5'-C4'	1.39		C6-H6	1.09	1.10	C3-H3(B)	1.10		O10-C10	1.44	
C5'-H5'	1.08		C5'-H5'	1.08		C8-H8	1.08	1.10	C1'-C6'	1.39	1.41	C10-H10(A)	1.09	
C1'-C6'	1.40	1.39	C6'-C1'	1.40		C2´-H2´	1.08	1.09	C1'-C2'	1.39	1.35	C10-H10(B)	1.09	
C6'-H6'	1.08		C6'-H6'	1.08		C3´-H3´	1.08	1.16	C5'-C6'	1.39	1.39	C10-C11	1.52	

Table 1
Continuation.

Bond	Chrysin		Bond	Galangin		Bond	Pinocembrin		Bond	Pinostrobin		Bond	CAPE	
length	**Theor.**	**Exp.[a]**	**length**	**Theor.**	**Exp.[b]**	**length**	**Theor.**	**Exp.[c]**	**length**	**Theor.**	**Exp.[d]**	**length**	**Theor.**	**Exp.[e]**
C1'-C2'	1.40	1.39	C1'-C2'	1.40		C4´-H4´	1.08	1.07	C6'-H6'	1.08		C11-H11(A)	1.09	
C2'-C3'	1.39	1.39	C2'-C3'	1.39		C5´-H5´	1.08	1.15	C4'-C5'	1.39	1.34	C11-H11(B)	1.09	
C2'-H2'	1.08		C2'-H2'	1.08		C6´-H6´	1.08	1.08	C5'-H5'	1.08		C11-C12	1.51	
C3'-C4'	1.39	1.39	C3'-C4'	1.39		C2-O1	1.43	1.39	C3'-C4'	1.39	1.40	C12-C17	1.40	1.39
C3'-H3'	1.08		C3'-H3'	1.08		C9-O1	1.36	1.37	C4'-H4'	1.08		C12-C13	1.40	
C4'-H4'	1.08		C4'-H4'	1.08		C5-O5	1.33	1.36	C2'-C3'	1.39	1.40	C16-C17	1.39	
O4-H5	1.67		H6'-O3	2.16		C7-O7	1.35	1.35	C3'-H3'	1.08		C17-H17	1.08	
O5-H5	0.99		O4-H5	1.76		C4-O4	1.23	1.27	C2'-H2'	1.08		C15-C16	1.39	1.38
O7-H7	0.96		O5-H5	0.98		O5-H5	0.99	1.09	O5-H5	0.96		C16-H16	1.08	
			O7-H7	0.96		O7-H7	0.96	1.10	O7-C11	1.42	1.40	C15-C14	1.39	
			O3-H3	0.97		O4-H5	1.70		C11-H(A)	1.09		C15-H15	1.08	
									C11-H(B)	1.09		C13-C14	1.39	1.38
									C11-H(C)	1.09		C14-H14	1.08	
												C13-H13	1.08	
												O2-H2	0.96	
D.S.	**0.000**						**0.004**			**0.001**			**0.000**	

Bond length in (Å)
Theor. Data obtained by M05–2X/6-31G(d,p).
[a] Taken from Ref. [Shoja M, 1989]
[b] Reference not showed
[c] Taken from Ref. [Guanhua et al., 2010]
[d] Taken from Ref. [Yamovoi, Kul'magambetova, Kulyyasov, Turdybekov, & Adekenov, 2001]
[e] Taken from Ref. [Son, Lobkowsky, & Lewis, 2001]

Table 2
Thermochemical and reactivity properties computed for phenolic compounds in gas–phase.

Compound	Property	HAT (Kcal/mol)			SPLET (Kcal/mol)					SET-PT (Kcal/mol)				
	Group	BDE		ΔE R	PA		ETE		ΔE A	IP		PDE		ΔE RC
Chrysin	5–OH	106[a]	96[b]	12	358[a]	349[b]	61[a]	62[b]	12	176[a]	176[b]	243[a]	235[b]	11
	7–OH	94	86	0	339	332	48	69	0			231	224	0
	8–CH	120	111	25	389	382	65	44	25			257	250	26
Galangin	3–OH	86	77	0	342	335	57	56	0	174	177	225	215	0
	5–OH	102	93	16	348	340	67	68	16			241	231	16
	7–OH	94	85	9	334	326	73	74	9			233	224	9
	8–CH	120	112	34	386	379	47	48	34			260	250	35
Pinocembrin	2–CH	85	76	0	373	365	25	26	36	183	183	216	208	0
	5–OH	104	95	20	356	348	61	62	18			235	226	20
	7–OH	106	96	10	338	331	81	80	0			236	228	10
	8–CH	120	111	34	394	386	39	40	56			251	243	34
Pinostrobin	2–CH	84	76	0	377	370	20	21	0	182	182	216	209	0
	5–OH	91	83	7	346	337	59	61	7			223	216	7
	8–CH	117	108	32	388	381	42	42	32			249	241	32
CAPE	2–OH	86	34	12	354	346	48	2	12	177	178	225	170	22
	3–OH	76	11	0	331	324	58	2	0			213	148	0

ΔE R= radical energy – neutral molecule energy.
ΔE A= anion energy – neutral molecule energy.
ΔE RC= radical cation energy – neutral molecule energy.
[a] Values from enthalpy.
[b] Values from free energy.

Table 3
Chemical potential properties for phenolic compounds in gas-phase.

Property (eV)	*A*	*I*	χ	η	ω
Chrysin	0.669	8.4	4.534	3.865	2.66
Galangin	0.808	8.048	4.428	3.62	2.708
Pinocembrin	-0.292	8.548	4.128	4.42	1.928
Pinostrobin	-0.311	8.418	4.054	4.365	1.883
CAPE	0.224	8.126	4.175	3.951	2.206

A= electronic affinity, *I*= ionization potential, χ= electronegativity, η= hardness, ω= electrophilicity.

Table 4. Chemical reactivity sites of phenolic compounds obtained by Fukui indices.

Compound	Atom	Nucleophilic attack	Atom	Electrophilic attack	Atom	Radical attack
Chrysin	H (7)	0.5080	O (7)	0.3114	H (6)	0.2835
Galangin	H (4')	0.2961	O (7)	0.3064	H (5')	0.3585
Pinocembrin	C (4)	0.1207	C (8)	0.1760	C (8)	0.2127
Pinostrobin	H (4')	0.2852	O (1)	0.2015	H (3)	0.3536
CAPE	H (15)	0.2791	O (9)	0.2441	H (15)	0.3229

CONCLUSIONES GENERALES

A partir de los resultados derivados de este trabajo de investigación se concluye que:

1. La incorporación de extractos etanólicos de propóleos (2%, p/p) en hamburguesas de bovino y de cerdo sin cocinar, conservadas en refrigeración (2 °C, en oscuridad), redujo significativamente (P<0.05) las reacciones de oxidación de lipidos y proteínas, así como la degradación del color. Estos resultados demuestran el potencial de los extractos de propóleos como ingrediente conservador durante el almacenamiento en refrigeración.
2. El origen botánico de las muestras de propóleos colectadas en las comunidades de Rancho Viejo y Pueblo de Álamos (Ures, Sonora) fue *Prosopis velutina* y *Mimosa distachya var. laxiflora*, respectivamente. Estas especies de plantas son caracteristicas del Desierto de Sonora y posiblemente son las que más contribuyan en la formación y producción de otros productos apicolas de la región. Ademas, los resultados demuestran que el origen botánico de 6 muestras evaluadas es bi-floral (15-45%), mientras que dos son multi-floral.
3. Las muestras de propóleos con altos contenidos de *Prosopis velutina* presentaron *in vitro* alto potencial antioxidante, asi como el mayor efecto frente a patogenos Gram-positivos (*S*. *aureus* y *L*. *inocua*), principalmente en las muestras colectadas durante el verano. Por otra parte, *Prosopis velutina* y *Mimosa distachya* var. *laxiflora* fueron efectivas frente a bacterias Gram-negativas (*E. coli* y *S. typhimurium*), principalmente durante la epoca de invierno (P<0.05).
4. Los compuestos identificados en todos los extractos etanólicos de propóleos fueron ácido gálico, ácido cinámico, ácido p-cumárico, naringenina, quercetina, luteolina, kaempferol, apigenina, pinocembrina, pinobanksina 3-

acetato, éster fenetílico del ácido cafeico, crisina, galangina, acacetina y pinostrobina.

5. Las propiedades fisicoquimicas, organolepticas y biológicas del propóleos estan correlacionadas con las coordenadas de color del producto en crudo, principalmente con el parametro h*.

6. Los funcionales PBEPBE, PBE1PBE, MPW1PW91, B3LYP y M05-2X en combinación con sus respectivos conjuntos de base [6-31G(d,p) y 6-31+G(d,p)] permitieron reproducir la estructura de pinocembrina, establecer su potencial químico y propiedades de reactividad. La química modelo M05-2X/6-31G(d,p) proporcionó la mejor calidad para simular la estructura de pinocembrina. Los valores bajos de potencial químico, obtenidos por las diferentes químicas modelos, indican que pinocembrina es capaz de donar electrones y participar como agente antioxidante, mientras que la reactividad analizada mediante los índices de Fukui muestran que los grupos $C_{(4)}$ fueron los sitios preferidos para el ataque nucleofílico, y los grupos $O_{(4)}$ y $C_{(8)}$, participaron en el ataque electrofílico y radical de la molécula.

7. El mecanismo dominante de inhibición de radicales libres de compuestos fenólicos (crisina, galangina, pinocembrina, pinostrobina y CAPE), en fase de gas, fue el HAT. El ácido fenólico (CAPE) fue el compuesto con mayor potencial antioxidante. Los resultados muestran que además de los grupos O-H, los grupos del carbono bencílico (C-H) contribuyen en la actividad antioxidante de los compuestos fenólicos encontrados en los EEP, tales como pinocembrina y pinostrobina. Se encontró que estos compuestos tienen mayor tendencia a donar electrones, y los sitios reactivos preferenciales de las moléculas estudiadas están situados en los anillos B y C.

ANEXOS

PRODUCCIÓN ACADEMICA

Artículos Publicados con Arbitraje

Rey David Vargas-Sánchez, Gastón R. Torrescano-Urrutia & Armida Sánchez-Escalante. El Propóleos: conservador potencial para la industria alimentaria (2013). ***Interciencia*** *38(10): 705-711*.

Vargas-Sánchez RD, Torrescano-Urrutia GR, Mendoza-Wilson AM, Vallejo-Galland B, Acedo-Félix E, Sánchez-Escalante JJ, Peñalba-Garmendia MC, & Armida Sánchez-Escalante Mechanisms involved in the antioxidant and antibacterial activity of propolis (2014). ***Biotecnia*** *16(1):32-37*.

Vargas-Sánchez, R. D., Torrescano- Urrutia, G. R., Acedo-Félix, E., Carvajal-Millán, E., González-Córdova, A. F., Vallejo-Galland, B., María J. Torres-Llanez & Sánchez-Escalante, A. (2014). Antioxidant and antimicrobial activity of commercial propolis extract in beef patties. ***Journal of Food Science***, *79*(8), C1499-C1504.

Estancias Científicas

Estancia en el laboratorio de (SARA) de la Universidad Veracruzana (UV), bajo la dirección del Dr. Javier Hernández Martínez.

Cursos

Asistencia al curso de "Técnicas de Palinología". Centro de Investigación Científica de Yucatán, A.C. Mérida, México. 2011.

Presentación en congresos Internacionales

Vargas Sánchez, R., Javier Sáiz, E., Torrescano Urrutia, G.R., Acedo Félix, E., Carvajal Millán, E., González Córdova, A.F., Vallejo Galland, B., Torres Llánez, M.J. Antioxidant and antimicrobial properties of commercial propolis in beef patties. IFT Annual Meeting. New Orleans, USA. 2011.

R.A. Kancab, E. Javier, **R.D. Vargas-Sánchez**, M.C. Estrada Montoya, B. Vallejo-Córdoba, A.F. González-Córdoba, E.A. Avila Espinosa, A.F. Varguez Pech, G. Torrescano-Urrutia, A. Sánchez-Escalante. Development of restructured beef using microbial transglutaminase and almonds as functional ingredients. IFT Annual Meeting. Las Vegas, USA. 2012.

Vargas-Sánchez RD, Torrescano-Urrutia GR, Mendoza-Wilson AM, Vallejo-Galland B, Acedo-Félix E, Sánchez-Escalante JJ, Peñalba-Garmendia MC & Sánchez-Escalante A. Antioxidant activity of bovine and porcine beef patties treated with propolis extracts. IFT Annual Meeting. Chicago, USA. 2013.

Zavala-Cárdenas L., Heredia B., Torrescano-Urrutia GR., **Vargas-Sánchez RD**., & Sánchez-Escalante A. Effect of addition of moringa flour in beef patties on the retardation of oxidation and quality changes during chilled storage. IFT Annual Meeting. Chicago, USA. 2013.

Salmerón-Ruiz ML, Gutiérrez-Ayuso B, Javier-Saiz E, **Vargas-Sánchez RD**, Ramírez-Rojo MI, Anaya-Islas J, Torrescano-Urrutia GR, & Sánchez-Escalante

A. Addition of pecans in beef patties as a functional ingredient. IFT Annual Meeting. New Orleans, USA. 2014.

Presentación en congresos nacionales

Vargas-Sánchez R.D., Mendoza-Wilson A.M., Torrescano-Urrutia G.R., Sánchez-Escalante Armida. Mecanismos involucrados en la actividad antioxidante de compuestos fenólicos encontrados en extractos de propóleos. Segundo Encuentro de Jóvenes Investigadores en el estado de Sinaloa. Mazatlán, Sinaloa. México. Septiembre, 2014.

Torres-Martínez B.M., **Vargas-Sánchez R.D**., Torrescano-Urrutia G.R., Sánchez-Escalante Armida. Evaluación de la actividad antioxidante de extractos de harina de Ganoderma. Segundo Encuentro de Jóvenes Investigadores en el estado de Sinaloa. Mazatlán, Sinaloa. México. Septiembre, 2014.

Salmerón Ruiz, M.L., Gutiérrez Ayuso, B., Javier Sáiz, E., **Vargas Sánchez, R.D**., Torrescano Urrutia, G.R., Sánchez Escalante, A. Desarrollo y evaluación de un producto cárnico funcional fresco tipo hamburguesa de bovino adicionado con nuez. 5o. Coloquio Nacional en Ciencia y Tecnología de la Carne. UAM Iztapalapa. Cd. México, D.F. 2011.